

STUDIES ON RNA FROM LIVER AND MAMMARY GLAND TISSUES

**EXTRACTION, FRACTIONATION, RNA METABOLISM
AND RNA-MEMBRANE INTERACTIONS**

BY

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C O N T E N T S

Summary	1
Publications	4
Introduction	5
Extraction of RNA	10
Regulation of the RNA content of cells by the action of RNAase and RNAase inhibitor	18
Chromatography of RNA on Sepharose	26
RNA-membrane interactions	32
Acknowledgements	40
Reference list	41

SUMMARY

A sequential extraction procedure using naphthalene-1,5-di-sulphonate (NDS) and phenol in the first step followed by a subsequent extraction of the RNA remaining in the insoluble interface with 4-aminosalicylate and triisopropyl naphthalene sulphonate (PAS-TIPNS) was studied (I). Approximately 80% of the extractable RNA from rabbit liver was recovered using NDS and treatment of the resulting interface with PAS-TIPNS released the remaining RNA into the aqueous phase. Subcellular fractionation and polyacrylamide gel electrophoresis or sucrose density gradient centrifugation showed that the PAS-TIPNS extracted RNA contained primarily 28S RNA from membrane-bound polysomes. Poly(A) containing RNA was retained in the phenol phase upon NDS treatment and was extracted with PAS-TIPNS.

The RNA extraction method was also studied using rabbit mammary glands (II). During early pregnancy the mammary alveolar cells contain only small amounts of membrane-bound polysomes. After delivery the rough endoplasmic reticulum is developed and consequently a high proportion of membrane-bound polysomes is found, whereas inhibition of the secretion of milk by ligation causes a decrease in the amount of membrane-bound polysomes. Most of the RNA was released into the aqueous phase upon NDS treatment when pregnant and lactating mammary glands were examined, whereas a large part of the RNA from lactating mammary glands was retained in the phenol phase and was subsequently extracted by PAS-TIPNS. Throughout the methodological studies of RNA extraction it was observed that both the pro-

portion of PAS-TIPNS extracted RNA and the total amount of RNA recovered from mammary glands varied during the three functional states. High amounts of RNA were found in the lactating glands and considerably lower amounts were found in the other two states, which imply that the level of RNAase and RNAase inhibitor is different during the physiological states.

Therefore, an investigation of RNAase and RNAase inhibitor was performed. A correlation was found between the amount of RNA and the activities of RNAase and RNAase inhibitor. During lactation the high RNA content was accompanied by a low RNAase and a high RNAase inhibitor activity. Upon ligation or during pregnancy the reversed pattern was seen, i.e. a low RNA content and a high RNAase and a low RNAase inhibitor activity.

Earlier studies have demonstrated a separation of rRNA (as one peak) from other nucleic acids on Sepharose 2B using Mg^{2+} in a buffer of low ionic strength at $22^{\circ}C$. By starting the chromatography of rRNA at $4^{\circ}C$ a resolution of 28S and 18S RNA was achieved (III). At that temperature 18S RNA was eluted first followed by a fraction of 28S RNA well separated from the 18S RNA peak. By increasing the temperature or removing Mg^{2+} from the medium an additional 28S RNA fraction was eluted. The relative amounts of 28S RNA and 18S RNA from NDS and PAS-TIPNS extracted RNA, were compared by this chromatographic technique. The NDS extracted RNA gave a chromatographic profile with approximately 30% of the RNA recovered in the 18S

RNA peak. This peak was very small in PAS-TIPNS extracted RNA, in accordance with the observation on polyacrylamide gel electrophoresis that PAS-TIPNS extracted RNA contains mainly 28S RNA.

Purification of poly(A) binding proteins from membranes of rapidly sedimenting endoplasmic reticulum (RS-ER) and microsomes by affinity chromatography on poly(A)-Sephadex was studied (IV). Approximately 2% of the proteins solubilized by Triton x-100 bound to the column and could be eluted by salt. SDS-polyacrylamide gel electrophoresis of the poly(A)-Sephadex eluate showed that RS-ER contained two poly(A) binding proteins with molecular weights of 54 000 and 79 000 d while microsomes contained four proteins with affinity for poly(A) with molecular weights of 60 000, 62 000, 63 000 and 79 000 d. By coupling the poly(A) binding proteins from RS-ER to Sephadex, the mRNA binding capacity of the purified proteins was studied. mRNA of lactating mammary glands adsorbed to the protein-Sephadex column. The mRNA was desorbed by an increase in the ionic strength and the in vitro translation was studied.

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I A Sequential Extraction Method of RNA from Rabbit Liver.
Bengt Axelsson, Allan Norgren, Lennart Holmqvist and Adam Deutsch (submitted for publication)
- II RNA Metabolism in Rabbit Mammary Gland During Different Physiological States.
Bengt Axelsson, Per-Erik Månsson, Allan Norgren and Adam Deutsch (submitted for publication)
- III Separation of rRNA on Sepharose 2B at Low Ionic Strength.
Bengt Axelsson, Björn Svensson and Adam Deutsch (submitted for publication)
- IV Preparation of Poly(A)-binding Proteins from Endoplasmic Reticulum - Containing Subfractions of Rat Liver Cells and Their Use in mRNA Purification.
Bengt Axelsson, Rolf Ohlsson, Adam Deutsch and Bengt Jergil
Mol. Cell. Biochem. (1977) 15, 67-71.

INTRODUCTION

Nucleic acids are polymers composed of a five carbon sugar, a heterocyclic nitrogenous base and phosphate as the monomeric unit (nucleotide) and these units are linked together with phosphodiester bonds to form the polymeric structure (polynucleotides). Two types of nucleic acids are found in the cell: the ribose containing ribonucleic acid (RNA) and the deoxyribose containing deoxyribonucleic acid (DNA). The bases found in the nucleic acids are purines and pyrimidines. In DNA the purines are guanine (G) and adenine (A) and the pyrimidines are cytosine (C) and thymine (T). The three first mentioned bases are also found in RNA, where uracil (U) is present instead of thymine.

DNA consists of two helical polynucleotides which are coiled around the same axis to form a double helix. The bases are oriented against each other inside the helix and form base pairs where A always pairs to T and G to C. Two polynucleotides are said to be complementary, when these base pairing rules are fulfilled.

The complementariness which is found in DNA is also found in RNA but to a much smaller extent. As RNA exists as single stranded molecules the complementary polynucleotide stretches are formed within the molecule. Thymine is exchanged to uracil in RNA and consequently the possible base pairs formed in order to achieve complementariness are G to C and A to U.

Nucleic acids in living organisms function as storage and transmitters of the genetic information. The expression of

the genetic material in eukaryotic cells involves transcription of the DNA template to form RNA in the nucleus. The transcribed products are then transported across the nuclear membrane to the cytoplasm where they are able to direct the incorporation of amino acids into proteins in an order determined by the nucleotide sequence of RNA.

Proteins are involved in all physiological processes in the cell and can be said to be the ultimate expression of the genes which was initiated in the nucleus by transcription. Since proteins are involved in so many different reactions in the cell, the transcriptional event is a major selective mechanism for controlling the protein content and thereby various functions of the organism.

Transcription of DNA to RNA is catalyzed by the enzyme DNA dependent RNA polymerase, where DNA is the template and the synthesized single stranded RNA molecule is complementary to one of the DNA strands. Eukaryotic nuclei contain a group of RNA polymerases of different function. They are classified according to their sensitivity to α -amanitin, a fungitoxin (1,2). RNA polymerase I, which is located in the nucleolus (3) is not affected by the toxin and is responsible for the synthesis of ribosomal precursor RNA (pre rRNA) (4), whereas RNA polymerase II is inhibited by very small concentrations of α -amanitin (1) and is located in the nucleoplasm. This enzyme is responsible for the synthesis of a population of RNA molecules of various molecular weights called heterogeneous nuclear RNA (HnRNA). A third enzyme, RNA polymerase III, catalyzes the synthesis of precursor transfer RNA (pre tRNA)

and 5S RNA which is an RNA species found in ribosomes.

The primary transcription products do not function in the protein synthesis system. They must be converted to functional units through complex maturation reactions called processing. The maturation of RNA involves nucleolytic cleavage, addition of nucleotides, methylation of ribose and bases and modification of the 5'-end of some molecules (capping).

Pre-tRNAs have been isolated from many eukaryotic cells and have a sedimentation coefficient of 4-5 S (5). The processing of pre-tRNAs to mature tRNAs involves methylation of some bases which might be a nuclear event (6) and removal of 15-35 nucleotides (7) which has been suggested to take place in the cytoplasm (7,8).

The processing of pre-rRNA occurs in the nucleolus. The transcript has a sedimentation coefficient of 45S with a molecular weight of 4.5×10^6 . The initial stage of processing involves methylation of the 45S RNA molecule (9). Most of the methylation reactions occur on the 45S RNA but some methylations occur during later stages of the processing (9) and even when the RNA has reached the cytoplasm (10). It is believed that methylation of the ribose at the 2'-position minimizes the risk for accidental nucleolytic cleavage of exposed phosphodiester bonds, as the methylation makes it impossible to form the cyclic 2'-3'-intermediate (11). Methylation of the bases is thought to be a means whereby loops

required for the proper function of rRNA are stabilized (12).

The transcribed 45S RNA unit contains the two ribosomal RNAs found in the cytoplasm (18S and 28S rRNA) surrounded by so called spacer sequences (13). The nucleolytic cleavage of 45S RNA is polar, starting from the 5'-end. The first step involves the hydrolysis of spacer material which gives an intermediate exposing the 5'-end of the 18S rRNA molecule. There are two possible routes to obtain the mature 18S RNA, which is the next step of the processing (13). The first possibility is a nucleolytic attack between the 3'-end of 18S RNA and the spacer segment, and the other is hydrolysis within the spacer sequences and a subsequent cleavage resulting in the mature 18S RNA. The last step in the processing pathway resulting in the mature 28S RNA involves two cleavages of the polynucleotide chain. A small fragment, 5.8 S RNA, located within the spacer segment between 18S RNA and 28S RNA is bound to the mature 28S RNA by base pairing after the final cut of the spacer sequences. Finally, throughout the processing of rRNAs proteins form a ribonucleoprotein structure with the pre-rRNA molecules (14). These proteins are synthesized in the cytoplasm and subsequently transported to the nucleolus. They are both ribosomal structural proteins and additional protein molecules and are thought to protect the pre-rRNAs from accidental hydrolysis (15).

Heterogeneous nuclear RNA (HnRNA) is a class of RNAs with a molecular weight varying between 10^5 and 2×10^7 . HnRNA has been suggested to be precursor of mRNA although this has not

been finally proven. The base composition of HnRNA resembles that of mRNA and there is a heterogeneity in molecular weight of both types of RNAs. It has been shown in HeLa cells that mRNA can hybridize to DNA which is complementary to Hn RNA (16), indicating that one part of the HnRNA molecule contains mRNA. Another piece of evidence for a relationship between HnRNA and mRNA is the fact that a polyadenylated chain of 150 - 200 nucleotides, added posttranscriptionally to the 3'-end, is found in both HnRNA and mRNA (17,18). As these homopolymers are found at the 3'-end from both types of RNA, it has been suggested that the mRNA sequence is located close to the 3'-end of the HnRNA molecule (19). But the finding of blocked 5'-termini ("caps", general structure $m^7GPPP N^m$) in both HnRNA and mRNA indicates that mRNA species may be located at the 5'-end of the HnRNA as well (20). The presence of dsRNA (double stranded RNA) (21) and oligo(U) and oligo(A) stretches (22,23) in HnRNA but not in mRNA, have been proposed to be recognition sites for enzymes involved in the processing of HnRNA. The processing pathway of HnRNA has not been finally settled, however.

EXTRACTION OF RNA

Several methods for the extraction of RNA from cells and tissues have been presented but ever since the discovery by Gierer and Schramm (24) that tobacco mosaic virus kept its infectivity after extraction with a water-phenol mixture, extraction with phenol has been the most commonly used method. Applying the phenol extraction method to mammalian cells makes the extraction more complex, since there are a number of RNA species which have different extraction properties in the two-phase water-phenol system. Thus, the question arises to what extent the extraction procedure can be elaborated to reproducibly yield various RNA species.

The isolation of specific RNA species can be achieved using two general approaches: either by extraction of subcellular fractions isolated by differential centrifugation, or by extraction of whole tissue followed by a fractionation of the nucleic acids. The latter method is preferable if a large quantity of RNA is to be prepared, since losses of RNA which occur upon subcellular fractionation is avoided. One of the problems encountered on extraction of unfractionated tissue is that it may result in contamination by macromolecules with extraction properties similar to the RNA species of interest and this may complicate further analyses of the RNA.

Subcellular fractionation of the tissue prior to extraction is preferable if RNA is prepared in a small scale or if RNA from a specific organelle is investigated. But there is a great problem associated with the fractionation. Once the

cells are disrupted by homogenization a liberation of RNAase occurs. This is a serious problem for all kinds of tissues and especially of RNAase rich tissues like pancreas (25). A degradation can be limited to some extent, if the fractionation is carried out in the cold and if the fractionation is done as quickly as possible. Another way to avoid RNA degradation is to add RNAase inhibitors in the fractionation buffer. The obvious advantages and disadvantages using either of the two approaches for RNA extraction have led to the development of sequential phenol extraction methods which make a fractionation of different RNA classes possible without a prior subcellular fractionation.

The phenol extraction method introduced by Georgiev and associates (26,27) is based on the fact that different types of RNA are extracted at different temperatures. Thus, extraction of RNA from whole cells by using a combination of a NaCl solution at weakly acidic conditions and phenol permits a thermal separation of both rRNA and mRNA, and also of pre-rRNA and pre-mRNA. Markov and Arion (28) have studied the Georgiev extraction method in great detail and their results clearly demonstrate the general problems which are associated with the phenol extraction procedure. They found that the composition and purity of the thermal fractions were strongly dependent upon extraction conditions as pH of the buffer, the time of thermal treatment, the ratio between extraction volume and tissue weight and even such a dubious point as the intensity of shaking during the thermal extraction procedure. The fact that pH changes affect the extraction conditions

for RNA has made it possible for a separation of the two large molecular weight rRNA species from cytoplasmic fractions (29). The extraction is started in a slightly acidic solution which will release 28S RNA. When temperature is increased there is a subsequent release of 18S RNA.

The thermal fractionation of RNA is one of the most commonly used phenol fractionation methods for isolation of specific RNA species. Another often used method introduced by Brawerman (30,31) is based upon a differential release of RNA into the aqueous phase at various pH values. By starting the extraction at neutral pH in the presence of sodium dodecylsulfate (SDS) and phenol, non-poly(A) containing RNA was released into the aqueous phase. A subsequent increase in pH of the extraction medium released the retained RNA into the aqueous phase. We have also achieved a purification of poly(A) containing RNA through a sequential extraction of rabbit liver tissue (I) using naphthalenesulphonate and phenol in the first step where the poly(A) containing RNA was retained in the phenol phase. The RNA was extracted in a second step by triisopropylnaphthalene sulphonate and 4-aminosalicylate (32). The retention of poly(A) containing RNA in the phenol phase at neutral pH can be ascribed to the poly(A) sequence itself indicated by the fact that all mRNA is retained with one exception, namely histone mRNA (33) which is known to lack that segment (34,35). The reason why poly(A) containing RNA is not released into the aqueous phase under those conditions is partly due to the ionic strength of the buffer used. Thus, the addition of water to the phenol phase after treatment at neutral pH will release the poly(A) containing RNA, whereas inclusion of NaCl in

the extraction buffer at an alkaline pH will decrease the extraction of poly(A) containing mRNA into the aqueous phase (36). RNA retained in the phenol phase can also be recovered by the addition of chloroform before reextraction (33). It was found that RNA obtained in this manner contained fragments which had an electrophoretic mobility similar to the poly(A) chain released from mRNA upon treatment with RNAase at high ionic strength. It appears that a stable poly(A)-protein complex rather than poly(A) alone has a high affinity for the phenol phase, since very little mRNA will be found in the phenol phase if polysomes are treated with proteases prior to the extraction. As the poly(A)-protein complex is found in the interface while the rest of the mRNA molecule is expected to orient into the aqueous phase, Perry et al (33) have proposed that the cleavage of poly(A) stretches is due to shearing forces during the phase separation procedure.

We have used the RNA extraction method introduced by Kirby and collaborators (32,37,38) for the preparation and fractionation of nuclear and cytoplasmic RNA from rabbit liver and mammary glands. The organic mixture used contained besides phenol, m-cresol (prevents phenol to crystallize at low temperature and acts as an additional deproteinizer) and 8-hydroxyquinolin (prevents phenol to be oxidized and chelates Mg^{2+}). The aqueous solution used in combination with the organic one contained naphthalene-1,5-disulphonate (NDS, inhibits RNAase). After homogenization of rat liver tissue in the NDS solution a phenol-cresol mixture was added and it was

shown that cytoplasmic RNA then was released into the aqueous phase (32). Nuclear RNA (identified as rapidly labelled RNA) and DNA remained as a precipitate between the two phases and was released into the aqueous phase by the addition of a solution containing triisopropyl naphthalene sulphonate (TIPNS, inhibits RNAase), 4-aminosalicylate (PAS, chelates Mg^{2+}), NaCl (increases the ionic strength in order to avoid denaturation of the RNA) and 2-butanol (solubilizes TIPNS, which is salted out in the presence of NaCl).

Further studies of the Kirby extraction procedure have shown, however, that there is an incomplete extraction of cytoplasmic RNA when NDS and phenol-m-cresol is used (I). Thus, extraction of RNA from rabbit liver showed that a part of 28s RNA from membrane-bound fractions was retained in the interface upon NDS treatment. This RNA could be released into the aqueous phase when the PAS-TIPNS solution was added to the insoluble interface.

The extraction conditions for RNA were also studied in rabbit mammary glands during different physiological states (II). During early pregnancy mammary alveolar cells contain little rough endoplasmic reticulum, but after delivery the proportion of this structure increases. When the secretion of milk proteins from the mammary glands is inhibited, which can be induced by ligation of the main excretory ducts, the amount of rough endoplasmic reticulum decreases again compared to lactation. When glands of these three functional states were sequentially extracted for RNA, it appeared that most of the

RNA from glands of pregnant rabbits and ligated glands was extracted with NDS, whereas a notable fraction of RNA from lactating mammary glands was retained in the phenol phase. The retained RNA was released into the aqueous phase after addition of PAS-TIPNS to the insoluble interface.

The higher proportion of PAS-TIPNS extracted RNA found in lactating mammary glands compared to ligated glands or glands of pregnant rabbits indicated that the RNA obtained by the subsequent treatment with PAS-TIPNS after NDS extraction was derived from membrane-bound polysomes, according to the discussion above.

Furthermore, hormone treated male rabbits which were supplemented with prolactin gave a proportionally higher amount of PAS-TIPNS extracted RNA from the mammary gland compared to those animals who did not receive prolactin (Table 1). As prolactin, a lactogenic hormone, is known to induce a selective increase in the number of polysomes bound to the membrane in mammary glands of pseudo-pregnant rabbits (39), these results suggest a similar increase in polysomes in the prolactin treated animals recorded as the higher yield of PAS-TIPNS extracted RNA.

It has been shown that EDTA removes the small ribosomal subunits and partly the large subunits from microsomal membranes prepared from rat liver and HeLa cells (40-42). Furthermore, it appears that the large ribosomal subunit binds to the membrane via specific membrane proteins (43). It seems possible,

TABLE 1

Sequential extraction of RNA from mammary glands of castrated and hormone treated rabbits.¹⁾

Treatment	NDS	PAS-TIPNS
	ODU ₂₆₀ /mammary gland	
Oestrone + Progesterone	29	0.5 (1.7%)
	32	0.3 (0.9%)
Oestrone + Progesterone + Prolactin	40	3.6 (8.5%)
	88	4.5 (4.9%)

¹⁾ Four castrated male rabbits were treated with optimal doses of oestrone and progesterone for 28 days. Two animals received 100µg prolactin twice daily from day 23 of the steroid hormone treatment. Two animals were not treated with prolactin and served as controls. The extraction was done sequentially using naphthalene-1,5-disulphonate (NDS) in the first step followed by a subsequent treatment of the insoluble interfaces with 4-aminosalicylate plus triisopropyl naphthalene sulphonate (PAS-TIPNS). The values within parentheses show the percentage of PAS-TIPNS extracted RNA of total RNA.

therefore, that the 28S RNA fraction which is retained in the organic phase upon NDS treatment of rabbit liver might be the EDTA resistant membrane-bound large ribosomal subunit fraction described above, and that it is retained when detergent is absent in the first extraction step. We have attempted to extract the EDTA resistant membrane-bound large ribosomal subunit sequentially with NDS and PAS-TIPNS in order to test whether such a correlation exists, but no conclusive results were obtained as the extracted material appeared to be heavily degraded.

Isolation and fractionation of RNA by the use of different phenol extraction methods are empirical procedures, based upon the observations that RNA shows different extraction properties when parameters like pH, temperature, detergent, salt, and chelating agents are varied. There are obvious problems to design an extraction procedure for any given RNA species in order to obtain a reproducible yield of that RNA. This means that there can be difficulties to evaluate the results obtained when RNA is extracted with the phenol procedure. This is of particular importance when RNA metabolism is studied under different physiological conditions. Regarding the extraction method introduced by Kirby (32, 37), such problems exist, if cytoplasmic RNA from tissues with varying amounts of rough endoplasmic reticulum are to be studied, since membrane-bound 28S RNA tends to be retained in the phenol phase upon extraction with NDS. If membrane-bound 28S RNA is to be investigated, the sequential extraction method could be a suitable method for its isolation.

REGULATION OF THE RNA CONTENT OF CELLS BY THE ACTION OF RNAase AND RNAase INHIBITOR

The rate of RNA synthesis and degradation in a cell differs widely depending on the need of proteins to be synthesized. For instance, changes in the metabolic state are accompanied by changes in the rate of hydrolysis of RNA. The dramatic effect on protein synthesis caused by hydrolysis of RNA is easily demonstrated by the fact that the breakage of only one diester bond in a mRNA molecule is sufficient for inactivation of its template activity and possibly leading to a disaggregation of polysomes into non-synthesizing ribosomal monomers. The susceptibility of mRNA to nucleolytic attack is also obvious since it is a large molecule with an average molecular weight of 500 000 which contains around 1000 phosphodiester bonds. The degradation of RNA is catalyzed by ribonuclease (RNAase). More than 80 different nucleolytic enzymes have been described (44), and in order to group these enzymes they have been classified according to their optimal activity at different pH values (45,46). This criterion must, however, be used with precaution since pH optima vary with experimental conditions such as concentration of urea and ionic strength (47-49). An acid RNAase (pH optimum 5.8) is localized in lysosomes, whereas an alkaline RNAase (pH optimum 7.8) has been found in several subcellular fractions. A third type of RNAase associated with the polysomes has been described. This nuclease is active at alkaline pH, but differs from the other enzymes by its heat lability and its inability to degrade poly(C), poly(A), poly(G) and poly(I) (50).

The activity of alkaline RNAase is restricted by a specific inhibitor which has been found in the cytosol (51). This inhibitor is capable of preventing degradation of high molecular weight nuclear RNA (52) and dissociation of polyosomes (54). The inhibitor is thermolabile, non-dialysable and is blocked by sulfhydryl reagents. It exists normally in excess in the cell and consequently the inhibitor and the RNAase form an enzymatically inactive complex. The inhibitor which is thought to be a glycoprotein (55) has been purified on DEAE-Sephadex and by affinity chromatography on RNAase-CM-cellulose (56). Its chemical composition has not been completely elucidated.

Several investigators have studied the activity of RNAase and RNAase inhibitor in tissues of different metabolic states as their activities are regarded as important regulatory factors in protein synthesis. Thus, Kraft and Shortman (57) found that lymphocytes from human peripheral blood, which has a low RNA content, showed no RNAase inhibitor activity and consequently the RNAase was in the free and active form (not complexed with the inhibitor). Stimulation of the lymphocytes with phytohaemagglutinin (a mitogen) transformed the cells to protein synthesizing blast cells. The transformation was accompanied by an increase in the amount of RNAase inhibitor whereas the RNAase activity remained at the same level as in resting lymphocytes.

The study of RNAase and RNAase inhibitor in rat liver has been performed during different functional states since the enzym system is very well characterized in that tissue.

Shepard et al (58) noticed that the amount of acid stable RNAase (extracted with 0.1 M H_2SO_4) increased during fasting, a state when rRNA degradation exceeds rRNA synthesis (59). In regenerating rat liver (60) the amount of RNAase inhibitor increased and remained at a high level for several days, while alkaline RNAase did not show any change in activity. These results imply that an increase in inhibitor activity is correlated with an active protein synthesis. The increase in activity of acid RNAase, on the other hand, occurred after the inhibitor had reached its maximum, and was suggested to be another mechanism whereby protein synthesis could be decreased.

Variations in the activity of RNAase and RNAase inhibitor in rat liver caused by hormonal influences have also been described. Thus, Brewer et al (61) showed that the size of polysomes as well as the capacity to support cell-free protein synthesis decreased in hypophysectomized rats as compared to non-treated ones. The RNAase level increased twofold in the hormone deficient animals. Treatment of the hypophysectomized animals with growth hormone returned the RNAase level as well as protein synthesis and the size of polysomes to normal. In livers from pregnant rats the same RNAase and RNAase inhibitor pattern was found as in regenerating rat livers (60). It has been proposed that this change to the regenerating pattern is connected with the regulation of mRNA coding for certain proteins (for instance serum albumin) present in an elevated level during pregnancy (62). It is interesting to note that these changes do not occur in livers from pregnant rabbits compared to lactating rabbits (II).

Mammary alveolar cells undergo a process of differentiation during pregnancy, lactation and involution which is controlled by several hormones. At early pregnancy the ribosomal population of mammary glands shows a significant amount of monomers and oligomers up to hexamers, while heavier polysomes constitute only a minor part (63). During this latter physiological state the ribosomes exist mostly as free polysomes (64). When lactation is initiated the most dominating polysomal structures are hepta- and octamers, and most of the polysomes are attached to the endoplasmic reticulum (II, 65). During involution, which can be experimentally induced by ligation of the mammary ducts during lactation, the proportion of membrane-bound polysomes decreases dramatically (II).

There is not only a variation in the polysomal composition of mammary cells during the three physiological states but there is also a change in the amount of RNA (Table 2, II, 66), indicating changes in the protein synthesis activity during the lactation cycle. The mechanism which regulates the amount of RNA during the cycle is not known in detail, but it is likely that both RNA synthesis and RNA degradation are closely regulated. Concerning RNA synthesis Banerjee and Banerjee (67,68) observed that ^3H -uridine was incorporated into RNA in mice mammary glands of pregnant and lactating animals whereas it was not incorporated in virgin mice. Furthermore, nucleoli of mammary gland cells of lactating mice incorporated more labelled precursor than did cells of pregnant animals. The nucleoplasm, however, showed

a similar incorporation in the two states, indicating a higher proportion of rRNA synthesis during lactation than during pregnancy. Table 2 confirms this observation. The table shows that only 45% of the total RNA content in pregnant rabbit mammary glands is rRNA while 72% of the total RNA is rRNA at lactation. It has been shown that a preferential stimulation of rRNA synthesis occurs by administration of a variety of hormones to different types of tissues

(69). Therefore, the differing proportions of rRNA in mammary glands during pregnancy and lactation may be due to hormonal stimulation of the synthesis of specific types of RNA at different stages of the development and function of the tissue. As lactation proceeds the rate of incorporation of nucleotides into RNA decreases (70) even though the concentration of RNA in the cell continues to increase (71). This indicates that there is a connection between RNA polymerase, RNAase and RNAase inhibitor activities which will control the amount of RNA in mammary glands during the lactation cycle.

TABLE 2

Comparison of the amount of total RNA, DNA and rRNA content in rabbit mammary glands during pregnancy, lactation and ligation of the main excretory ducts at lactation

	Total DNA mg/g tissue	Total RNA mg/g tissue	Total rRNA mg/g tissue
Pregnant	2.5 ± 0.9	3.1 ± 0.7	1.4 ± 0.2 (45%)
Lactating	2.6 ± 1.0	10.0 ± 3.1	7.2 ± 1.5 (72%)
Ligated	2.6 ± 0.1	5.3 ± 1.3	3.7 ± 1.3 (70%)

The calculations of total RNA and DNA were performed according to the Schmidt-Tannhauser-Schneider procedure as described by Volkin and Cohn (72) and the rRNA content is expressed as the sum of naphthalene-1,5-disulphonate and 4-aminosalicylate plus triisopropyl naphthalene sulphonate extracted RNA (I) from whole tissue homogenates. Subtractions of nuclear and mRNA in the rRNA fractions have not been done. The values within parentheses show the percentage of rRNA of total RNA.

We have studied the amount of RNA, RNAase and RNAase inhibitor activities in rabbit mammary glands at different functional states during the lactation cycle (II). Our aim for this investigation of RNA metabolism in mammary glands during different functional states was to study whether the activity of RNAase and RNAase inhibitor varied at different stages of the lactation cycle and to examine whether there was a correlation with the amount of RNA found in the tissue. Furthermore, by inhibiting the secretion of milk by unilateral ligation of the main excretory ducts the possibility arose to study whether the hindered secretion of proteins caused changes in the RNAase and RNAase inhibitor patterns even though the hormonal level was directed towards lactation and production of proteins. It was found that the RNAase activity was lower during lactation than during pregnancy while the inhibitor activity and the RNA content were higher. Unilateral ligation of the main excretory ducts caused a decrease in the inhibitor activity and an increase in the RNAase activity. As a consequence of this a decrease in the RNA content was found.

In rats Slater (73) found the highest alkaline RNAase activity at lactation. The specific activity at this stage was almost twice as high as during pregnancy, and it decreased again during involution to approximately 50% of the activity found during lactation. These results contradicts ours, where lactation showed significantly lower activity than both pregnancy and after ligation (II). The results of Slater can to some extent be explained by the fact that he did not include p-chloromercuriobenzoate, which will inactivate the

inhibitor, in his assay. Then most of the RNAase was complexed with the inhibitor during the assay.

On the other hand, the findings of Liu et al (74) concerning rat mammary glands are in general accordance to ours, i.e. high inhibitor/RNAase ratio at lactation compared to pregnancy and involution. Even though there was a decrease in this ratio after ligation compared to lactation the effect was more marked at weaning. As the effect of RNAase is the same during weaning (74) and ligation (II) but the effect of inhibitor is more marked during weaning, it seems that the inhibitor effect is not completely expressed in mammary glands unless the involution is influenced by other factors than inhibition of the secretion of milk caused by ligation. The RNAase and RNAase inhibitor pattern in rabbit mammary glands (II) and in the same tissue of rats (74) indicates that the increase in inhibitor activity and the decrease in RNAase activity occur during active protein synthesis and thus acts as a regulator for the production of protein as has been found in other tissues.

Chromatography of RNA on Sepharose.

Various column chromatography methods have been employed for separation of nucleic acids. Compared to other separation techniques (for example ultracentrifugation (75), electrophoresis (76,77) and counter current distribution (78)) chromatography by gel filtration or on ion exchange resins is rapid, simple and permits a preparative isolation of the desired RNA species. The use of column chromatography for separation of RNA is advantageous not only from a practical point of view; chromatographic behaviour of RNA might also reveal chemical and biological characteristics which are not detected by other separation techniques.

Anion exchangers have been useful in resolving RNA from both prokaryotic and eukaryotic cells, since RNA exists as highly negatively charged molecules at neutral pH. The most commonly used anion exchangers have been DEAE cellulose and DEAE-Sephadex. Important factors which influence the resolution of RNA on these matrices are pH, temperature, type of column matrix and urea concentration used (79). Using these variables three principles for separation of RNA can be discerned: length, of the RNA chain (inclusion of urea or variations in temperature will change the separation pattern), fraction of protonated bases (a decrease in pH will increase the proportion of protonated bases and thus change the separation pattern) and interaction with the matrix (purines interact with cellulose but not with dextran (80)).

Ion exchange chromatography is appropriate for fractionation of tRNA. This RNA class is homogeneous in molecular weight

and molecular sieving, therefore, is less useful.

The separation of tRNA has been improved further by the discovery of Gillam et al (81) that esterification of hydroxyl groups on DEAE cellulose by the aromatic benzoyl group (BD-cellulose) changed the resolution profile of tRNA compared to unsubstituted DEAE cellulose; benzoyl residues increased the affinity for aromatic and hydrophobic groups (82).

Ion exchange chromatography has not been used extensively for separation of rRNA species. However, Parish (83) resolved rat liver 18S and 28S RNA components on DEAE-cellulose in an isopropanol-triethylammonium acetate buffer. It was also shown that 4S RNA was eluted at a low ionic concentration followed by 18S and 28S RNA, indicating that molecular size is one of the factors that influence the separation of RNA species on DEAE-cellulose. Other techniques than ion exchange chromatography have been employed for the separation of rRNA species. Thus, reversed phase chromatography (RPC) has been shown to be very versatile for resolving E.coli rRNA species. The technique is based on differences in partition coefficient of different RNA species between two partly miscible liquid phases, using a more hydrophobic phase as the stationary phase and a hydrophilic one as the moving phase. 16S and 23S RNA were resolved by using a linear salt gradient in the RPC system (84). Liver 18S and 28S RNA could not be separated by this method, however.

Sephadex 2B and 4B are commonly used for separation of RNA of different classes. Sephadex contains agarose (an alternating copolymer of galactose and anhydrogalactose) and varying amounts of agaropectin, which is a collective designation for polysaccharides containing sulfate and carboxyl groups. Agar is commonly used for gel filtration since it has a rigid structure even at a concentration of 1%. The rigidity of agar at low concentrations permits it to be used as a bed material for filtration of high molecular weight compounds when the less rigid dextran and polyacrylamide can not be used (85). The exclusion limits for dextran and polyacrylamide are below 5×10^5 and these materials can only be used to separate tRNA from high molecular weight RNA species (86).

Gel filtration on agarose has been shown to be versatile for separation of different species but has certain limitations. Thus, Öberg and Philipson (87) have separated total KB cell nucleic acids and polio virus RNA on 1% agarose at low ionic strength with Mg^{2+} in the elution medium at $22^{\circ}C$. They demonstrated that DNA was excluded from the column whereas the RNA species were eluted according to molecular weight, i.e., the viral RNA (mw 2×10^6) was eluted first followed by rRNA (as one peak) and tRNA. By increasing the concentration of agarose the viral RNA (3% agarose) and rRNA (4% agarose) appeared in the void volume. These factors strongly indicate that gel filtration on agarose is possible using buffer conditions which avoid interactions between nucleic acids and agarose.

Contrary to these findings about gel filtration of high molecular weight RNA species, low molecular weight RNA species have been selectively resolved on unsubstituted Sepharose 2B (88). The total tRNA population was retained on Sepharose at a high $(\text{NH}_4)_2\text{SO}_4$ concentration at pH 4.5. Using a negative salt gradient tRNA species were separated. As the adsorption occurred at high ionic strength the nonpolar character of Sepharose seems to be involved in the binding to tRNA. Contradictory results were obtained by Dziekielewski and Jakubowski (89) who showed that tRNA was retained on amino-alkyl Sepharose at low ionic strength and subsequently eluted by an increase in the ionic strength.

DNA, rRNA and tRNA have been separated on Sepharose 4B using high salt at pH 7.5 (90). Chromatography of the nucleic acids resulted in a void fraction containing DNA and a fraction eluting around V_t which contained tRNA (tRNA is not retained on Sepharose at neutral pH) (see ref. 88), whereas rRNA was retained and subsequently eluted by lowering the salt concentration from 2.5 M to 0.1 M.

Separation of rRNA and tRNA at high salt concentration has also been done on Sephadex G 75 (91). At 1,5 M NaCl, tRNA appeared in the void volume whereas rRNA was retained and was subsequently eluted at a decreased ionic strength.

The fractionation of rRNA has been studied in more detail by Popovic (92) and Petrovic et al (93,94). By using an appropriate salt concentration, 28S RNA could be retained selectively on Sepharose at room temperature, whereas 18S RNA

eluted. 28S RNA could be eluted subsequently by decreasing the ionic strength of the medium or raising the temperature to 35°C.

In the present work separation of the large rRNA species was also demonstrated by chromatography on Sepharose 2B at low ionic strength with Mg^{2+} in the elution medium at 4°C (III). 18S RNA eluted first followed by a part of the 28S RNA. By subsequently excluding Mg^{2+} from the elution buffer or when the temperature was raised to 22°C the retained 28S RNA fraction eluted. A comparison of the elution patterns at 4°C and 22°C in the presence of Mg^{2+} showed that the elution position of 18S RNA was the same at the two temperatures whereas 28S RNA eluted after 18S RNA or was retained on Sepharose only at the low temperature. If the chromatography was performed at 22°C no separation of the rRNA components occurred. The finding that 28S RNA is retained on Sepharose in the presence of Mg^{2+} can be compared to the observations by Popovic (92) and Petrovic et al (93,94) showing the same effect in the absence of Mg^{2+} using high monovalent cation concentrations.

The reasons why rRNA is retained either completely or selectively on Sepharose at high monovalent cation concentrations are not clear. Several proposals have been made and the explanation might be one or a combination of the following factors. a) The conformational state of rRNA. This is implied by the observations that changes in monovalent cation concentrations or temperatures, factors which influence the stability

of the secondary structure of RNA, strongly affect the chromatographic behaviour of rRNA on Sepharose. b) Adsorption of RNA on Sepharose. This is supported by the observation that high molecular weight RNA but not rRNA or DNA can form a gel-like aggregation at high monovalent cation concentration (95, 96). c) Hydrophobic interactions between the hydrocarbon moiety of Sepharose and rRNA. The facts that 28S RNA contains a higher proportion of the hydrophobic nucleoside guanosine than 18S RNA and is more strongly bound to Sepharose at high salt, imply that hydrophobic forces are involved in the retention of rRNA to Sepharose.

The retention of 28S RNA on Sepharose 2B at low temperature and low ionic strength in the presence of Mg^{2+} can neither be explained in terms of gel formation between RNA and agarose nor in terms of hydrophobic interactions, since such interactions are not stabilized by Mg^{2+} in the absence of monovalent cations. An explanation might be that the divalent cations act as ionic bridges between the anionic polysaccharide and the negatively charged phosphate groups of 28S RNA.

RNA-MEMBRANE INTERACTIONS.

Ribosomes of mammalian cells are present in two morphologically and biochemically distinguishable states. One class exists free in the cytoplasm and the other is attached to the microsomal membranes, denoted as rough endoplasmic reticulum. The proportion of these two classes of ribosomes vary widely depending on the function of the cell. Thus, cells involved in the synthesis of proteins primarily designated for export like pancreatic acinar cells, plasma cells or cells of lactating mammary glands contain almost only ribosomes associated with the membrane, whereas rapidly multiplying cells as those from embryonic and tumor tissues with an active synthesis of cellular proteins contain a high proportion of free polysomes. This suggests that membrane-bound ribosomes are involved in the synthesis of secretory proteins whereas free ribosomes synthesize proteins which are retained in the cell.

Taking another approach for the elucidation of the functional differences between free and membrane-bound polysomes, there have been several studies on the subcellular localization of the synthesis of secreted and unsecreted proteins from the same tissue. Serum albumin (a secreted protein) and ferritin (a retained protein) have served as markers for the two types of proteins in liver. Over 90% of serum albumin is synthesized on membrane-bound liver polysomes (97), indicating that secreted proteins are synthesized on ribosomes attached to membranes. It serves to be mentioned that a majority of albumin synthesized in hepatoma cells is not secreted,

and is formed on free polysomes (98). Other secreted proteins which are preferentially synthesized on membrane-bound polysomes are γ -globulin (99), β -lactoglobulin (100) and serum glycoproteins (101).

Ferritin biosynthesis is rather evenly distributed between free and membrane-bound polysomes (102). This is not expected from the general concept that proteins for intracellular use are synthesized on free polysomes. Upon administration of iron (a prosthetic group of ferritin) a three-fold increase in ferritin synthesis was observed on free ribosomes, whereas membrane-bound polysomes did not respond on the addition of iron, indicating that ferritin synthesis on free polysomes is involved in the regulation of its synthesis. But still the reason for the presence of ferritin mRNA on membrane-bound polysomes is unclear. The biosynthesis of another soluble liver protein, serine dehydratase (103) occurs to a great extent on membrane-bound polysomes whereas a third soluble protein, arginase, has been shown to be preferentially synthesized on free polysomes (104).

Three mechanisms have been proposed by which polysomes are interacting with the membrane. They may interact via the large ribosomal subunit, by the growing peptide chain and/or via mRNA. These types of interactions might operate independently from each other and possibly represent classes of ribosomes with mRNA coding for different proteins. On the other hand, all types of interactions might operate on the same polysome and represent different stages of the translation. This raises the possibility that ribosome membrane association can act as a mechanism for the control of translation.

It has been shown that membrane-bound polysomes are attached to the membrane via the large ribosomal subunit (41). Treatment of rough microsomes from liver tissue with puromycin causes a dissociation of nascent peptide chains from the ribosome without a detachment of the large ribosomal subunit from the membrane (105). But if the treatment is carried out in the presence of high salt the large ribosomal subunit is released from the membrane (105,106). This suggests that a direct binding exists between the large subunit and the membrane which is stabilized by ionic interactions.

It has been shown, however, that a large portion of the 60S ribosomal subunit is detached from membranes following treatment with RNAase, EDTA or puromycin (92). This ribosomal fraction which might be anchored to membranes only via mRNA has been termed loosely bound ribosomes. Polysomes which contain 60S subunits that are unaffected by those treatments seem to be bound to the membrane via the large ribosomal subunits and are termed tightly bound polysomes. Using the definition by Adelman et al (105) and Harrison et al (106) the loose class includes ribosomes which are attached to the membrane only by the large ribosomal subunit (released by KCl) and the tight class includes polysomes also attached via the nascent peptide chain (released by puromycin and KCl). It has been proposed that loosely bound ribosomes are precursors of the tightly bound class, implied by the fact that the loosely bound fraction contains shorter peptide chains than the tight class (107). It has also been shown that the loosely bound ribosomal fraction contains ribosomal monomers whereas the tight

class is constituted of heavier polysomal structures (108).

Tata (109) has suggested that there is a difference in the mechanism of vectorial discharge of nascent polypeptides synthesized on polysomes attached to membranes which takes into account that non-secretory proteins are also synthesized on membrane-bound polysomes. He proposed that the nascent chain of non-secreted proteins is passed through a tunnel of the large ribosomal subunit and canalized along the endoplasmic reticulum before release into the cytoplasm upon completion of synthesis. The nascent chain of secreted proteins instead penetrate the microsomal membrane and enters the cisternal space.

An explanation for the topographical selectivity of mRNA coding for secreted proteins has been given by the work of Blobel and Dobberstein (110). They studied the translation of IgG light chain with mRNA from murine myeloma cells in a heterologous cell-free protein synthesizing system with free ribosomes. The translated IgG light chain had a molecular weight of 25 000 and was larger by approximately 4000 d than the light chain secreted from the myeloma cells. Translation of the light chain mRNA using dog pancreatic rough microsomes instead of free polysomes yielded the native IgG light chain with a molecular weight of 21 000. The extra 20 amino acids at the N-terminus is thought to be a signal sequence with which peptides destined for secretion from the cell will penetrate the membrane and thereby anchor polysomes to the membrane. Before the translation is completed the signal sequences will be removed by proteolytic attack. The codon for the

extra amino acids (signal codon) is placed immediately after the initiation AUG-triplet at the 5'-terminus of mRNA. Other studies from Blobel's laboratory have shown similar signal sequences of unsecreted prolactin (pre-prolactin), growth hormone (pre-growth hormone) from bovine anterior pituitary glands (111) and insulin (pre-pro-insulin) from fish (112).

mRNA has been proposed to play a role in the attachment of polysomes to the membrane (113). Treatment of membrane-bound polysomes of rapidly sedimenting membrane structures from human fibroblasts with puromycin in high salt (114) and HeLa cells with EDTA (113) will cause an incomplete detachment of mRNA from the membrane. Furthermore, when the unreleased mRNA was digested with RNAase, the RNA was hydrolyzed except the RNAase resistant poly(A) sequence which remained attached to the membrane. This indicates that the point of attachment is on or close to the poly(A) segment of the mRNA. Milcarec and Penman (113) have suggested that proteins associated with the poly(A) segment could be responsible for the interaction between membrane and mRNA. There have been attempts to isolate such specific proteins. Thus, mRNP particles released from membrane associated polysomes of Erlich ascites tumor cells contained one protein complexed with the poly(A) moiety, but the same protein was also found in free mRNP-particles (115).

This indicates that proteins associated with the poly(A) segment of mRNA will not selectively associate with the membrane. However, direct interactions between proteins of the endoplasmic reticulum and the poly(A) chain itself may exist. Thus, when endoplasmic reticulum containing subfractions of rat liver were examined for their existence of poly(A) binding proteins approximately 2% of the solubilized membrane proteins showed affinity for poly(A)-Sephadex 4B (IV). Two membrane fractions were investigated, rapidly sedimenting endoplasmic reticulum (RS-ER) and microsomes. Concerning RS-ER, one protein with a molecular weight of 54 000 which bound selectively to poly(A)-Sephadex was found. The microsomal fraction appeared to have three such proteins with molecular weights of 57 500, 60 000 and 63 000. The existence of different sets of poly(A) binding proteins in the two membrane classes might indicate that there are different mechanisms for the interaction between ribosomes and membranes in RS-ER and microsomes. Another piece of evidence for different interactions between the polysomes and the two membrane fractions is that a cytochrome P-450-ribosome complex has been found in RS-ER but that it is absent in microsomes (116). Furthermore, the two fractions differ with respect to their relative contents of mRNA coding for secreted proteins (117).

Two identifiable types of rough endoplasmic reticulum exist in rat liver. One type consists of stacks of parallel cisternae found in the perinuclear region of the cell (RS-ER) while the other type (rough microsomes) exists as single cisternae found throughout the cytoplasm. The differences between the two ER-fractions are primarily based upon electron microscop-

pical observations. Therefore, it is only possible to speculate about the functional significance of the various sub-fractions of the ER. The observation that RS-ER is present close to the nucleus raises the possibility that the 54 000 d protein found in RS-ER might act as a receptor for mRNA which has newly entered the cytoplasm. It has been demonstrated in yeast that newly synthesized poly(A) containing RNA is rapidly transported from the nucleus to cytoplasmic membranes. A release of membrane-bound mRNA was observed after the initiation of protein synthesis (118). If a similar mechanism is operating in rat liver, the poly(A) binding protein specific for RS-ER may act as a receptor for any newly synthesized poly(A)-containing mRNA. This would facilitate the binding of ribosomes to the endoplasmic reticulum as mRNA then is located close to the membrane prior to the initiation of protein synthesis and thus will increase the probability for nascent chain (the signal sequence) to recognize receptors on the membrane. Since a large fraction of non-secreted proteins is synthesized on free polysomes, the mechanism requires that a release from RS-ER of the mRNA takes place after initiation. The release might occur upon completion of the first polypeptide, i.e. when the corresponding ribosome is close to the poly(A) segment of the mRNA.

The presence of three proteins with affinity for poly(A) in the microsomal fraction suggests that different interactions between mRNA and the membrane exists. The function of these proteins might be to interact specifically with different types of mRNA or that they will act together to bind mRNA. Another possible explanation is that each poly(A) binding pro-

tein is localized in a specific subfraction of the microsomes. A further fractionation of the microsomal membrane might show if this is the case.

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TITLE: A Sequential extraction method for RNA from rabbit liver.

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ABSTRACT

When naphthalene -1,5- disulphonate (NDS) and phenol-cresol were used for extraction of rRNA from rabbit liver approximately 80% of the extractable RNA was recovered. By a subsequent treatment of the resulting insoluble interface with 4-aminosalicylate and triisopropyl naphthalene sulphonate (PAS-TIPNS) the remaining RNA was extracted. Subcellular fractionation and polyacrylamide gel electrophoresis or sucrose density gradient analysis showed that this last fraction contained 28s RNA from membrane-bound polysomes. Applying the sequential extraction method for isolation of poly(A)-containing mRNA showed that only a minor part of that RNA was extracted with NDS while the rest was recovered by PAS-TIPNS treatment of the interface. Thus, a purification of poly(A)-containing mRNA was possible when the sequential extraction method was used.

INTRODUCTION

Earlier studies have demonstrated a differential release of nucleic acids from mammalian tissues depending on the nature of the extraction agents used in the aqueous phase of the two phase phenol-water extraction system. Thus, the extraction of rat liver in disodium naphthalene 1,5-disulphonate (NDS) and a phenol-cresol mixture yields cytoplasmic nucleic acids, i.e. ribosomal and transfer RNA in the aqueous phase. On the other hand, all nucleic acids including DNA and rapidly labelled RNA are released into the aqueous phase if the extraction is carried out in a mixture of sodium 4-aminosalicylate (PAS), tri-isopropyl naphthalene sulphonate (TIPNS) and phenol-cresol (1,2). A combination of the two extraction procedures yields rRNA and tRNA in a first step using naphthalene disulphonate, while DNA and rapidly labelled RNA are extracted from the resulting insoluble interface with 4-aminosalicylate and tri-isopropyl naphthalene sulphonate (2).

When we applied this sequential extraction procedure for the isolation of rRNA we found contrary to earlier reports (2) that a significant amount of rRNA was not extracted by NDS and phenol. This rRNA remained in the insoluble interface of the extraction system and could be released by a subsequent treatment with PAS-TIPNS.

Here we have examined closer the extraction conditions for rabbit liver RNA, especially rRNA and poly(A)-containing RNA using the sequential extraction procedure. A significant part of 28s RNA remains insoluble upon NDS extraction and can be released by a subsequent PAS-TIPNS treatment. Furthermore, a considerable part of poly(A)-containing RNA remains in the phenol phase upon NDS treatment and is extracted subsequently into the aqueous phase with PAS-TIPNS.

MATERIALS AND METHODS

MATERIALS

^3H -Adenosine and ^{35}S -methionine were purchased from The Radiochemical Centre, Amersham. Sodium tri-isopropyl naphthalene sulphonate and disodium naphthalene 1,5-disulphonate were obtained from Eastman Kodak Co, Rochester, New York and poly(U)-Sepharose from Pharmacia Fine Chemicals, Uppsala. ATP, GTP, creatine phosphate, creatinephosphokinase, HEPES, and spermine were from Sigma Chemical Co, St. Louis.

METHODS

Fractionation of tissue

Albino rabbits of mixed strain, starved for 24 hours, were killed by cervical dislocation and exsanguinated. Livers were removed immediately and rinsed at 0° in buffer (0.05M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M MgCl_2 and 0.25 M sucrose). They were minced and homogenized in a Potter-Elvehjem homogenizer in two ml of buffer per g of tissue. The homogenate was centrifuged at 10000 xg for 10 min to sediment cell fragments, nuclei and mitochondria. The postmitochondrial supernatant was centrifuged in a Beckman 60 Ti rotor to sediment microsomes, either at 105000xg for 3 hours or over a discontinuous sucrose gradient (10 ml 2.0 M and 10 ml 0.5 M sucrose in buffer and 13 ml post-mitochondrial supernatant) for 4 hours at 250000xg. The pellet obtained at 105000xg, the material collected between 0.5 M and 2.0 M sucrose in the gradient (containing membrane-bound polyosomes) and the resulting pellet (free polyosomes) were used for subsequent experiments.

Extraction of RNA

An outline of the extraction procedure is presented in Fig. 1. Whole tissue or subcellular fractions were homogenized with 10 ml of 0.5% (w/v) naphthalene 1,5-disulphonate (NDS) per g wet tissue. Then 6.6 ml of a phenol solution (500 g phenol, 70 ml m-cresol,

0.5 g 8-hydroxyquinoline and 55 ml water) was added. The mixture was stirred for 30 min at room temperature before centrifugation at 10000xg for 10 min. The upper aqueous phase was removed and the phenol phase and the interface were washed twice with 0.5% naphthalene 1,5-disulphonate. The aqueous phases were combined and solid sodium tri-isopropyl naphthalene sulphate was added to a concentration of 5% followed by half a volume of the phenol solution described above. After stirring for 10 min the phases were separated by centrifugation at 10000xg for 10 min. The aqueous phase was made 10% in m-cresol, 3% in NaCl and 20% in sodiumbenzoate (m-cresol mixture) to precipitate the RNA except 4S and 5S RNA which remain in solution. The precipitate was washed with the m-cresol mixture followed by 1% NaCl in 75% ethanol, 75% ethanol and ethanol. RNA obtained in this manner will be called NDS-extracted RNA.

The phenol phase and the interface from the naphthalene 1,5-disulphonate extraction were treated with 10 ml per g wet tissue of a solution containing 6% sodium 4-aminosalicylate (PAS), 6% butanol (v/v), 1% NaCl and 1% tri-isopropyl naphthalene sulphate (TIPNS). After stirring for 30 min at room temperature the phases were separated by centrifugation (10000g per 10 min). The aqueous phase was made 3% in NaCl. Half a volume of the phenol solution was added and the mixture was stirred for 10 min. The aqueous phase was collected and two volumes of ethanol containing 10% m-cresol (v/v) were added. RNA and DNA were allowed to precipitate at -20°C for 1 h. The precipitate was washed twice at $+4^{\circ}\text{C}$ with 2% sodium acetate in 75% ethanol and was then dissolved in 0.1 M sodium acetate, pH 6.0. The solution was made 4 M in NaCl and RNA was allowed to precipitate over night at $+4^{\circ}\text{C}$. After centrifugation the RNA pellet was washed with 4 M NaCl in 0.1 M sodium acetate and dissolved in water. In some cases the pellet was further washed with 75% ethanol, ethanol and finally dried in a vacuum. RNA obtained in this manner will be called PAS-TIPNS extracted RNA.

Sucrose density gradient centrifugation

Approximately 150 μg of RNA in 100 μl sodium acetate buffer was applied on top of 4 ml linear 5-20% (w/v) sucrose gradients pre-

pared in 0.01 M sodium acetate, pH 5.2. Centrifugation was for 4 hours at $+4^{\circ}\text{C}$ at 35000 rpm in a Beckman SW-39 rotor. The gradients were eluted from the bottom of the tubes and monitored continuously at 254 nm. Fractions containing 4 drops were collected for radioactivity analyses.

Electrophoresis.

RNA: Mixed agarose-polyacrylamide gels (0.5% agarose, 2% total acrylamide monomer concentration, with a 5% proportion of N,N'-methylene-bisacrylamide) were prepared in a buffer consisting of 0.04 M Tris-HCl, pH 7.8, 0.02 M sodium acetate and 0.001 M EDTA (3). Five cm gels were polymerized in glass tubes, inner diameter 0.5 cm. Electrophoresis was for 35 min at 3-4 mA per tube at $+4^{\circ}\text{C}$ using the gel buffer in the electrode vessels. Samples containing 0.5 ODU₂₆₀ of RNA were layered over the gel. After the run, gels were placed in 1 M acetic acid for 15 min before staining for 1h in 0.2% methylene blue dissolved in 0.2 M sodium acetate and 0.2 M acetic acid. Excess stain was removed by rinsing in several changes of water.

Proteins: Sodium dodecylsulphate-polyacrylamidegel electrophoresis was performed in slab gels according to Neville (4) in a modified (R. Ohlsson, B. Jergil, personal communication) Pharmacia GR 4 apparatus. Radioactive material from in vitro protein synthesis (1.5×10^6 cpm) was applied to the gel. Electrophoresis was run at 20 mA per gel and was discontinued when the front reached the bottom of the gel. Gels were stained in 0.1% Coomassie Brilliant Blue dissolved in 50% methanol and 7% acetic acid and destained in 25% methanol and 7% acetic acid. Gels were dried under vacuum at $+100^{\circ}\text{C}$ and autoradiographed. The exposure was for 3 days using Osray T4 films.

Chromatography on poly(U)-Sephadex

Isolation of poly(A)-containing mRNA on poly(U)-Sephadex was performed according to Lindberg and Persson (5) in a 1 x 3,5 cm column (the binding capacity was 2.5 mg poly(A)). RNA (5-15 mg) was dissolved in water and diluted with 10-15 vol of 0.05 M Tris HCl, pH 7.5, 0.7 M NaCl, 0.01 M EDTA and 25% formamide before application to the column. Poly(A)-binding material was eluted

with 0.01 M Tris-HCl, pH 7.5, 0.01M EDTA, 0.2% sarcosyl and 90% formamide. The material was precipitated with 2 vol of ethanol and 0.1 vol of 20% potassium acetate, washed in ethanol, dried in a vacuum and dissolved in water.

In vitro protein synthesis

Translation experiments were carried out in a wheat embryo extract (6). The incubation mixture contained 1 mM ATP, 0.2 mM GTP, 8.8 mM creatine phosphate, 80 μ g/ml creatine phosphokinase, 2 mM dithioerythritol, 20 mM HEPES, pH 7.6, 64 mM KCl, 3.2 mM magnesium acetate, 16 μ M each of 19 amino acids (except methionine) 5 μ Ci 35 S-methionine (200-300 Ci/mMol), 30 μ g/ml spermine (7), 20 μ l S-30 extract (6) and mRNA purified from 50-500 μ g RNA in a total volume of 50 μ l. After incubation for 60 min at + 30°C 5 μ l aliquots were put on Whatman 00 filters and labelled proteins were precipitated in cold 10% trichloroacetic acid. The filters were washed by boiling for 10 min in the same solution, followed by 10 min in 5% trichloroacetic acid and 5 min each in ethanol and ether. The filters were dried and counted in a liquid scintillation counter.

Analysis of poly(A)-containing mRNA

RNA samples (2-5 ODU₂₆₀) labelled with 3 H-adenosine were dissolved in icecold 0.01 M Tris-HCl, pH 7.6, 0.5 M KCl and 0.001 M MgCl₂ and filtered slowly through Millipore filters (HA 0.45 μ m, Millipore Filter Corp., Bedford, Mass.), previously soaked in the same solution (8). The filters were heatdried before counting.

RESULTS AND DISCUSSION

Extraction of rRNA with NDS and PAS-TIPNS.

In preliminary experiments we found that the recovery of RNA using the NDS extraction method (1) was lower than with another method utilizing a pH 7.6 buffer in the presence of SDS and phenol (9). This indicates that an incomplete extraction of RNA occurs when NDS is used. Furthermore, treating the tissue directly with PAS-TIPNS gave a much higher recovery of rRNA than using NDS. These results encouraged us to study the sequential extraction method of Kirby in more detail. The method was first applied on unfractionated rabbit liver tissue (Table 1). In the first NDS step, 85% of the total extractable RNA was recovered, while the remaining RNA was extracted in a second step by PAS-TIPNS. In order to study the subcellular localization of the RNA extracted by PAS-TIPNS a fractionation of the liver cell in fractions containing nuclei (10000xg pellet) and microsomes was made prior to extraction. Of the total RNA extracted from these two fractions together approximately 40% originated in the microsomes. Both in the 10000xg pellet and in the microsomes 10-15% of the RNA remained insoluble upon NDS treatment and was extracted subsequently by PAS-TIPNS. Thus a significant proportion of the microsomal RNA remains unextracted by NDS and is recovered by PAS-TIPNS treatment contrary to the findings of Parish and Kirby (2).

We have also examined the distribution of radioactively labelled RNA between the 10000xg pellet and the microsomes, 20 min and 11 h after intraperitoneal injections of ^3H -adenosine (Table 1). After 20 min of labelling the specific radioactivity of RNA extracted by NDS was low in both fractions, while it was several times higher in the RNA extracted subsequently by PAS-TIPNS. Most of the label was found in the 10000xg pellet. After 11 h the label was equally distributed between the fractions. The difference in specific radioactivity between the RNA extracted by PAS-TIPNS and NDS was then less pronounced than after 20 min of incorporation.

In order to identify the RNA extracted by the sequential procedure the material was examined by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. Fig 2 shows the sucrose density gradient profiles of RNA extracted from the 10000xg pellet and microsomes which have been labelled for 20 min and 11h. After 20 min of labelling no distinct RNA peak was seen in the material extracted with NDS from the 10000xg pellet while radioactive material much heavier than 28s RNA was extracted by the subsequent treatment with PAS-TIPNS. Since the RNA extracted with PAS-TIPNS should be composed mainly of nuclear RNA (2) this is in accordance with earlier investigations. The amount of radioactive material incorporated into the microsomal fraction was not sufficient for a centrifugation analysis. After 11 h of labelling the gradient profiles in both the 10000xg pellet and the microsomes showed radioactive peaks corresponding to 28s and 18s RNA in both the NDS and the PAS-TIPNS extracts. In addition, radioactive material of a lower sedimentation rate than 18s was particularly conspicuous in the PAS-TIPNS extract of the microsomal fraction. This material which has a high specific radioactivity has not yet been identified but might be degraded mRNA. The material of the PAS-TIPNS extract sedimenting around 18s appears to contain labelled RNA other than 18s RNA. The ratio of 28s:18s RNA in the PAS-TIPNS extract is considerably higher than the expected 2.5:1 ratio seen for instance in the NDS extracts.

The RNA extracted from the microsomal fraction by NDS and consecutively by PAS-TIPNS was further analyzed by polyacrylamide-agarose gel electrophoresis (Fig. 3). As a control the microsomes were extracted with PAS-TIPNS without a prior NDS treatment. Again the PAS-TIPNS extract of the sequential procedure contained a higher proportion of 28s RNA than expected, while the NDS extract and the control extraction with PAS-TIPNS showed the expected 2.5:1 distribution between 28s and 18s RNA. Therefore the high 28s:18s ratio of the PAS-TIPNS extract obtained through the sequential procedure appears not to be due to artifacts of the preparation. More likely it is due to an incomplete extraction of 28s RNA in the NDS step leaving a significant proportion of the 28s RNA to be extracted by PAS-TIPNS, Since the amount

of RNA extracted by PAS-TIPNS compared to NDS is rather low (see Table 1), this extra 18s RNA in the NDS extract will not appreciably affect the RNA pattern.

In order to further examine the localization of the PAS-TIPNS extractable RNA, free and membrane-bound polysomes were isolated and extracted by the sequential method. While most of the RNA from free polysomes could be extracted with NDS (Table 2), approximately a third of the RNA from membrane-bound polysomes was retained in the phenol phase upon NDS treatment and was extracted into the aqueous phase during the following PAS-TIPNS treatment. When ribosomes were released from membrane-bound polysomes with deoxycholate prior to extraction all of the ribosomal RNA was extracted with NDS. It therefore appears that a sizeable fraction of the 28s RNA of membrane-bound polysomes is protected from NDS extraction by the membrane. The recovery of the RNA from free and membrane-bound polysomes was about 40% of the amount obtained in the unfractionated microsomes (cf Table 1). This was due to losses in the sucrose cushion which retains monosomes and ribosomal subunits on gradient centrifugation. All the RNA remaining in the sucrose cushion was extracted by NDS when the sequential method was applied. This is in line with the other results presented indicating that only membrane-bound RNA is left unextracted by NDS.

The choice of extraction method for RNA is very important to obtain optimal amounts of the RNA species intended for study. The results presented indicate that extraction with NDS and phenol-cresol according to Kirby (1) yields rRNA from free polysomes and most of the rRNA from small subunits of membrane-bound polysomes. On the other hand, the large ribosomal subunit of membrane-bound polysomes tends to be retained in the interface upon NDS extraction and the RNA is released by the addition of PAS-TIPNS. The reason for this retention of rRNA from membrane-bound large ribosomal subunits upon NDS treatment may have several explanations. The most likely explanation might be the tight association between the large ribosomal subunit and the endoplasmic reticulum (10,11) which appears to be mediated via specific proteins (12) or the other components which may stabilize the ribosome-membrane bind-

ing (13-15). The more efficient extraction obtained with PAS-TIPNS may be due to the strong hydrophobic nature of TIPNS compared to NDS, probably resulting in a detergent-like action which should aid in the detachment of the large ribosomal subunit from the microsomal membrane.

Extraction of poly(A)-containing RNA with NDS and PAS-TIPNS

The retention in the phenol phase of rRNA upon extraction with NDS, possibly due to interactions between ribosomes and the membrane, made us extend the study of RNA extraction to include mRNA. It is wellknown that mRNA exists as an mRMP, (free unit or bound to the membrane) (16,17). The proteins associated to the poly(A)-moiety of the mRNA or poly(A)-sequency alone, have been reported to cause an incomplete extraction of poly(A)-containing mRNA (8,18) when an aqueous phenol mixture is used. We therefore examined whether a preferential extraction of poly(A)-containing RNA would occur with NDS and PAS-TIPNS and whether a subfractionation of this RNA could be achieved by the sequential method. To follow the extraction, RNA was labelled radioactively by an injection of ^3H -adenosine 22 h before sacrifice of the animal. Unfractionated liver tissue and microsomes were extracted by the sequential method and poly(A)-containing RNA was selectively isolated by adsorption to Millipore filters (8). Table 3 shows the distribution of poly(A)-containing RNA from NDS and PAS-TIPNS extracted material. Even though about 30% of the total RNA label appeared in the PAS-TIPNS fraction there was a clear enrichment of poly(A)-containing material in that extract (65% of the total poly(A)-containing RNA). Considering the total amount of RNA in the extracts a fivefold enrichment of poly(A)-containing RNA was found in the PAS-TIPNS fractions compared to the NDS ones.

We tested whether different species of poly(A)-containing mRNA were extracted by NDS and PAS-TIPNS. The extracted RNA was purified on poly(U)-Sepharose 4B and was then used to direct protein synthesis in a wheat germ in vitro system. The radioactively labelled polypeptides were separated by SDS gel electrophoresis and autoradiographed (Fig 4). When the products of NDS and PAS-TIPNS extracted RNA were compared no significant differences were found in their polypeptide pattern. NDS treatment therefore seems insufficient for a complete extraction of the poly(A)-containing RNA rather than being a selective extraction step. However, NDS extraction prior to PAS-TIPNS appears to be

a useful step to remove large amounts of RNA, primarily rRNA, which otherwise will contaminate the mRNA preparation.

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TABLE 1.

Extraction of RNA from whole tissue and subcellular fractions with NDS and PAS-TIPNS

Fraction	RNA extracted	Incorporation time		
		20 min	11 h	
	ODU ₂₆₀ /g tissue	dpm/ODU ₂₆₀	ODU ₂₆₀ /g tissue	ODU ₂₆₀ /g tissue
Whole tissue				
NDS	84 ⁺ 12			
PAS-TIPNS	10 ⁺ 2			
10000xg pellet				
NDS	55 ⁺ 20	316	50	926
PAS-TIPNS	4 ⁺ 3	1987	4	3190
105000xg pellet				
NDS	27 ⁺ 8	65	35	3190
PAS-TIPNS	5 ⁺ 2	191	3	2200

TABLE 2

Recovery of RNA from free and membrane-bound polysomes sequentially extracted with NDS and PAS-TIPNS.

Fraction	RNA extracted (ODU ₂₆₀ /g tissue)			
	Experiment A ¹⁾		Experiment B ²⁾	
	NDS	PAS-TIPNS	NDS	PAS-TIPNS
Free polysomes	7.0	0.1	4.0	0.1
Membrane-bound polysomes	5.0	1.7	2.6	1.1
Membrane-bound polysomes after deoxycholate treatment			2.7	0.0

1)

A postmitochondrial supernatant was layered over a discontinuous sucrose gradient (see Materials and Methods) and centrifuged for 4 h at 250,000xg. Free and membrane-bound polysomes were collected and extracted.

2)

Isolated membrane-bound polysomes from a discontinuous sucrose gradient centrifugation were divided into two parts. One part was treated with 1% deoxycholate dissolved in postmicrosomal supernatant (to inhibit RNAase activity) and recentrifuged over a discontinuous sucrose gradient, while the other part was suspended in postmicrosomal supernatant prior to centrifugation. Free polysomes were also resuspended and recentrifuged.

TABLE 3

Sequential extraction of poly(A)-containing RNA from whole liver tissue and microsomes with NDS. Rabbits were injected with 0,5 mCi ^3H -adenosine 22 h before sacrifice. RNA was extracted by the sequential method. RNA samples (2-5 ODU₂₆₀) were applied on Millipore filters in the presence of 0.1 M Tris-HCl, pH 7.6, 0.5 M KCl and 0.001 M MgCl₂. The filters were washed in the same solution, heatdried and counted.

Fraction	Extraction agent	RNA extracted	Poly(A)containing
			RNA
			<u>dpm per g tissue</u>
Whole tissue	NDS	102672	1233
	PAS-TIPNS	37956	2204
Microsomes	NDS	31864	192
	PAS-TIPNS	13243	410

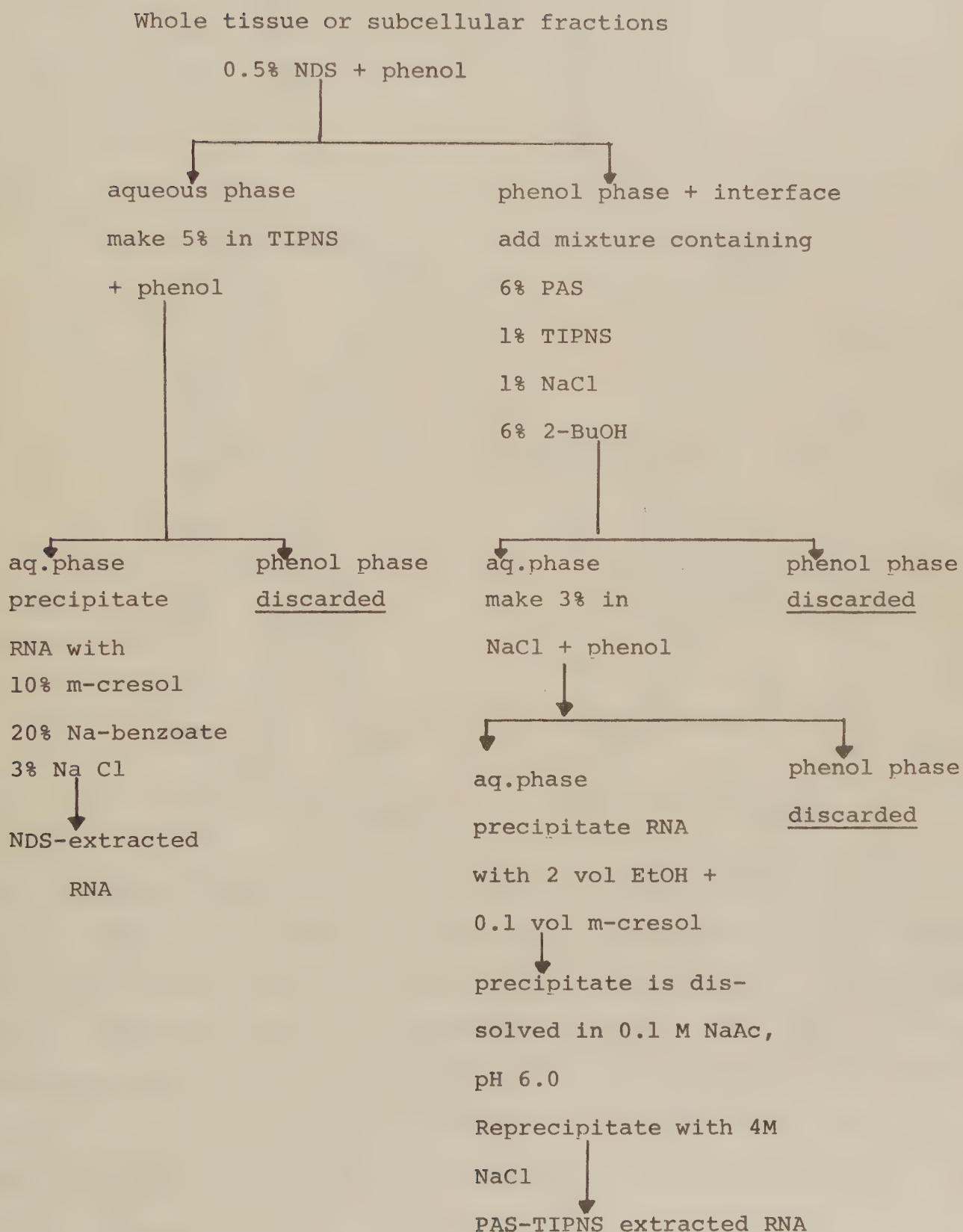


Fig. 1. Sequential extraction procedure for RNA.

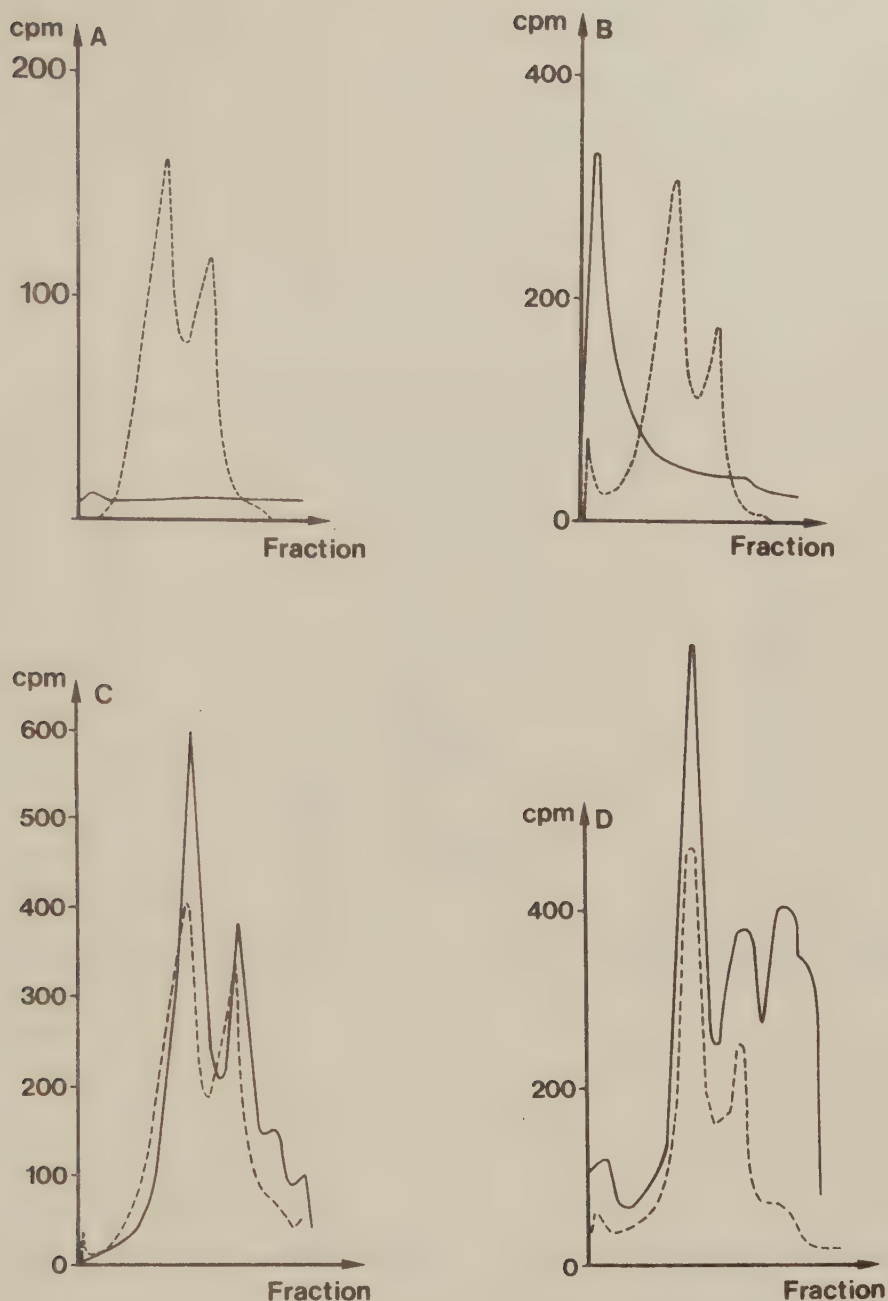


Fig. 2 Sucrose density gradients of RNA extracted by the sequential method. Rabbits were injected with 0.5 mCi ^3H -adenosine 20 min (nuclear containing pellet) and 11 h (microsomes) before sacrifice. Nuclear containing (10000xg) pellet extracted with A) NDS, B) PAS-TIPNS and microsomes extracted with C) NDS and D) PAS-TIPNS were run in 5-20% gradients for 4 h at 35000 rpm in a Beckman SW 39 rotor. Fractions of 4 drops were collected and counted in a liquid scintillation counter. — cpm
- - - - A_{254} .

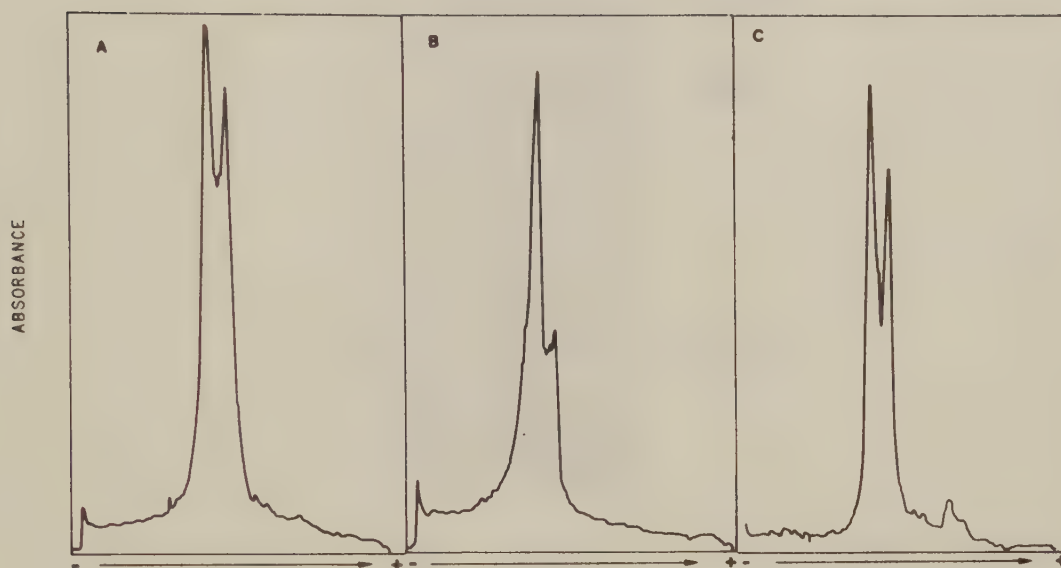


Fig. 3 Polyacrylamide-agarose gel electrophoresis of microosomal RNA sequentially extracted with A) NDS and B) PAS-TIPNS and C) direct extraction with PAS-TIPNS. Electrophoresis was performed as described in Material and Methods. The gels were photographed and the negative film was scanned in a densitometer.

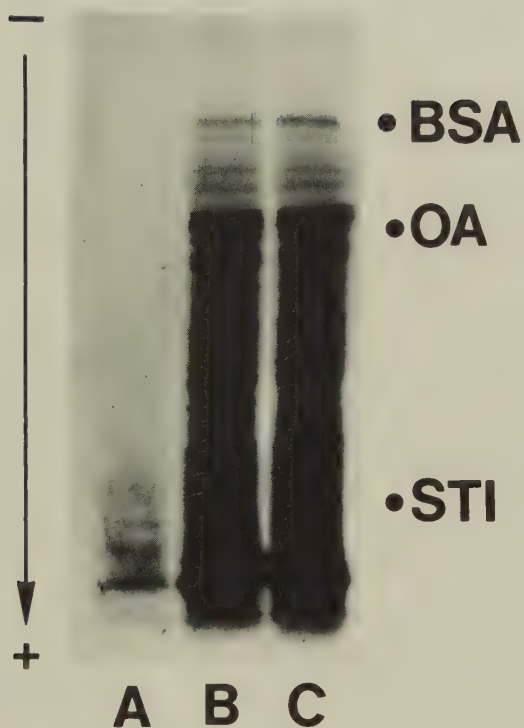


Fig. 4 SDS-polyacrylamide gel electrophoresis of polypeptides synthesized from microsomal mRNA extracted by the sequential method. After extraction the RNA was further purified by poly(U)-Sepharose chromatography. The polypeptides were labelled with ^{35}S -methionine. Electrophoresis was run as described in Material and Methods and was dried and autoradiographed. A) No mRNA added, B) mRNA from the NDS extract and C) mRNA from the PAS-TIPNS extract. BSA: Bovine Serum Albumin, OA: Ovalbumin and STI: Soybean Trypsin Inhibitor have been used as molecular weight markers.

TITLE: RNA Metabolism in Rabbit Mammary Gland During Different Physiological States

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ABSTRACT

Alkaline RNAase and RNAase inhibitor and the content of RNA in rabbit mammary glands were investigated during different physiological states. At mid-lactation the RNAase activity was lower than during pregnancy while the inhibitor activity and the RNA content were higher. Ligation of the main excretory ducts of the lactating glands caused a decrease in the inhibitor activity and an increase in the RNAase activity when compared to non-ligated glands. Ligation also caused a decrease in the RNA content.

The amount of membrane-bound polysomes was lower during pregnancy and upon ligation of the main excretory ducts of lactating mammary glands than in lactating glands.

INTRODUCTION

In most species studied, full development of the mammary alveolar system occurs only during pregnancy. When lactation is initiated further marked changes are observed. These include the production of proteins, lactose and lipids of milk. Such biological events are controlled by several hormones, among them ovarian hormones and prolactin. (1,2).

During early pregnancy mammary alveolar cells are rather uniform in structure, containing little rough endoplasmic reticulum and consequently small amounts of membrane-bound polysomes. After delivery, the structure is different. The endoplasmic reticulum of the rough type is now a prominent feature along with the secretion of milk (3). We have previously found (4) that when RNA is extracted from rabbit liver according to the sequential method of Parish and Kirby, (5,6) RNA from free polysomes and the small subunit of membrane-bound polysomes is extracted in a first step with naphthalene - 1,5-disulphonate (NDS) and phenol-cresol. A subsequent treatment of the resulting interface with 4-amino-salicylate and triisopropyl naphthalene sulphonate (PAS-TIPNS) yields additional RNA which consists mainly of 28s RNA from membrane-bound polysomes. We have examined here the extraction of RNA by the same procedure from rabbit mammary gland.

It is known that the RNA content of mammary cells increases when lactation is initiated (7,8), reflecting the increasing and different demands placed upon the mammary glands when the specialised proteins of milk are synthesised. This increase in RNA might be due to an in-

creased rate of synthesis and/or to a decreased rate of hydrolysis. Thus information about the levels of RNAase and RNAase inhibitor activity should be useful for an understanding of the RNA turnover in the cell.

From studies in liver tissue an RNAase and RNAase inhibitor system has been found which might be involved in the control of the cellular RNA content and thereby protein synthesis (9-12). In mammary glands a similar control system might be operating during different functional states. Thus, Liu et al (13) reported that in rat mammary glands an increase in the RNA content was accompanied by a decrease in alkaline RNAase and an increase in the alkaline RNAase inhibitor activity.

Two types of RNAase can be distinguished in mammalian tissue, an alkaline RNAase and an acid RNAase (14,15). The alkaline RNAase which most likely hydrolyses cytosolic RNA is found in various subcellular fractions including the cytosol and the microsomes. The acid RNAase appears to be restricted to the lysosomes as are several other hydrolases. An inhibitor for alkaline RNAase has been found in the cytosol (16,17).

This report is focused on the relationship between the amount of RNA and alkaline RNAase and its inhibitor in rabbit mammary glands during three different functional states, namely early pregnancy, mid lactation and when the emptying of the glands is inhibited on one side by ligation of the main excretory ducts. Ligation can to some extent be comparable to weaning although the hormonal control of the ligated tissue is the same as in the lactating tissue.

The amount of free and membrane-bound polysomes was also examined in the three functional states by subcellular fractionation of a post-mitochondrial supernatant and by the sequential extraction method using NDS in the first step followed by extraction of the insoluble interface with PAS-TIPNS (4).

MATERIALS AND METHODS

Animals and operation

Primiparous rabbits of a mixed strain were used and litters of less than six young were excluded. They were kept in separate cages and fed ad libitum with a pelleted diet and tap water.

Ligation of the main excretory ducts on one side was performed between day 14 and 19 of lactation, i.e. at the height of lactation. (18), in local anesthesia with Xylocain^R (Astra). The rabbits were sacrificed four days later by dislocation of the neck and exsanguinated. Pregnant rabbits were always sacrificed on day 14 of pregnancy. Milk, mammary glands and liver were removed rapidly and processed as soon as possible.

Fractionation of the tissue

All steps were carried out at +4°C. Rabbit liver and mammary glands were chilled immediately and rinsed carefully in cold buffer (0.05M Tris-HCl, pH 7.5, 0.025M KCl, 0.005M MgCl₂). For RNAase experiments the tissues were minced in an MSE glass-homogenizer in 2-4 volumes of 0.44M sucrose in the buffer and for RNA extraction studies in buffered 0.25M sucrose.

The homogenisation was completed with ten strokes in a Potter-Elvehjem homogenizer to give the homogenate. The homogenate was centrifuged at 15000xg for 10 min. The postmitochondrial supernatant was centrifuged in a Beckman 60 Ti rotor to sediment microsomes, either at 105000xg for 3 hours or over a discontinuous sucrose gradient (10ml 2.0M and 10ml 0.5M sucrose in buffer and 13ml postmitochondrial supernatant) for 4 hours at 250000xg. The pellet obtained at 105000xg, the material collected between 2.0M and 0.5M sucrose in the gradient (containing

membrane-bound polysomes) and the resulting pellet (free polysomes) were used for subsequent experiments. The 105000xg supernatant is referred to as the cytosol.

Extraction of RNA

The extraction of RNA was performed sequentially using naphthalene-1,5-disulphonate (NDS) and phenol-cresol followed by an extraction of the insoluble interface with 4-aminosalicylate plus triisopropyl naphthalene sulphonate (PAS-TIPNS) as described by Parish and Kirby (6). An outline of the extraction is illustrated in Fig. 1.

Electrophoresis

Mixed agarose-acrylamide gels (0.5% agarose, 2% total acrylamide monomer concentration with a 5% proportion of N,N' -methylene-bisacrylamide) were prepared in a buffer consisting of 0.04M Tris-HCl, pH 7.8, 0.02M sodium acetate and 0.001M EDTA (19). Five cm gels were polymerised in glass tubes (i.d. 0.5 cm). Electrophoresis was for 35 min at 3-4 mA per tube at +4°C using the gel buffer in the electrode vessels as coolant. Samples containing 0.5 ODU₂₆₀ of RNA were layered over the gel. After the run gels were placed in 1M acetic acid for 15 min before staining for 1 h in 0.2% methylene blue dissolved in 0.2M sodium acetate and 0.2M acetic acid. Excess stain was removed by rinsing in several changes of water.

Alkaline RNAase and RNAase-inhibitor

The assay procedures for RNAase and RNAase-inhibitor were essentially as described by Kraft and Shortman (20). A detailed description has been given elsewhere (21).

Total alkaline RNAase was measured at pH 7.8 in the presence of 0.001 M p-chloromercuribenzoate (PCMB) to inactivate the inhibitor.

Free alkaline RNAase was measured at pH 7.8 and analyses were performed using fresh homogenates in the presence of 0.005M EDTA to avoid heavy metal ions, which inactivate the inhibitor (22). Excess inhibitor was measured by the reduction in activity of a standard amount of added crystalline bovine pancreatic RNAase. One inhibitor unit is the amount which gave a 50% inhibition of 2 μ g of crystalline pancreatic RNAase. One RNAase unit is the amount which had the same activity at pH 7.8 as did 1 μ g of crystalline pancreatic RNAase.

Protein was determined according to the method of Lowry et.al. (23).

RESULTS

Sequential extraction of RNA

The sequential extraction method according to Parish and Kirby (6) was first applied to mammary glands of pregnant and lactating rabbits (Table 1). The amount of NDS-extracted RNA was three to four times higher in lactating mammary glands than in mammary glands of pregnant rabbits. When the extraction was carried further with PAS-TIPNS treatment, the difference in RNA yield between the functional states was even more marked (10-20 times). These differences indicate that there is an increased amount of membrane-bound polysomes in lactating mammary glands compared to glands of pregnant rabbits in accordance with the earlier investigation (4). Extraction of RNA from microsomes of lactating and ligated mammary glands also showed differences in the amount of NDS and PAS-TIPNS extracted RNA (Table 2). The yield of NDS extracted RNA from microsomes of ligated mammary glands was about half of the amount recovered from lactating glands.

There was also a decrease in the yield of RNA extracted by PAS-TIPNS following ligation. This was more marked than the decrease seen on NDS extraction indicating a decrease in the amount of membrane-bound ribosomes. It was not possible to compare the extraction of microsomal RNA from mammary glands of pregnant rabbits to the other two functional states, since the yield of RNA upon extraction was poor due to difficulties to properly homogenise this tissue.

The RNA extracted from the microsomal fraction of lactating mammary glands by NDS and PAS-TIPNS was also analysed by polyacrylamide-agarose gel electrophoresis (Fig. 2.) The PAS-TIPNS extract contained a

higher proportion of 28s RNA as expected, while the NDS extract showed the normal distribution between 28s and 18s RNA, in accordance with an earlier observation on rabbit liver (4).

The observed differences in the total amount of RNA and in membrane-bound polysomes during lactation and ligation was studied in more detail by fractionation of the microsomes of lactating and ligated glands in discontinuous sucrose density gradients. Postmitochondrial supernatants from lactating and ligated mammary glands of the same animal were fractionated into free and membrane-bound polysomes and monosomes plus ribosomal subunits (RNA from unsedimented ribosomes which appears in the 2M sucrose cushion) in order to compare the amount of RNA in these fractions during the different experimental states (Table 3). In lactating mammary glands the RNA recovered from membrane-bound polysomes dominates, while only traces of RNA were found in this fraction following ligation. On the other hand both free polysomes and monosomes plus ribosomal subunits increased markedly upon ligation.

From the experiments presented so far can be seen that there are great differences in the amount of RNA which can be extracted from mammary glands in the pregnant, lactating and ligated lactating states. This indicates that there are differences in the RNA metabolism in the various physiological states. To study a possible basis for these differences the presence of RNAase and RNAase inhibitor in mammary glands was examined using the same tissue preparations.

Alkaline RNAase

Free RNAase was analysed in the absence of PCMB while total RNAase was measured in the presence of PCMB to inactivate the RNAase inhibitor.

The amount of the total RNAase in the homogenate from one mammary gland increased when the animals passed from the pregnant to the lactating state (Table 4). The specific activity of RNAase, however, decreased threefold during the same period. Upon subfractionation of the homogenate a large fraction of the alkaline RNAase was recovered in the cytosol while small amounts sedimented with the microsomes. Most of the remaining activity was recovered among the material sedimenting at 15000xg (not shown in the table). When free alkaline RNAase was measured (i.e. analyses performed in the absence of PCMB) no activity was observed in lactating glands whereas at early pregnancy free alkaline RNAase activity corresponding to approximately 5% of the total alkaline RNAase was present in the cytosol. After ligation of the lactating glands the total alkaline RNAase activity and its specific activity increased compared to non-ligated glands. Furthermore, the specific activity was close to that of mammary glands during pregnancy. Free alkaline RNAase activity also increased upon ligation. Thus in the cytosol the specific activity was slightly higher than that found during pregnancy. As a control the total and free alkaline RNAase activities were measured in livers from pregnant and lactating rabbits (Table 4). No differences in total RNAase activity in the two physiological states could be seen and no free alkaline RNAase activity was detected.

Milk obtained from lactating rabbits showed a slightly lower level of free alkaline RNAase than that of total alkaline RNAase (Table 4). This indicates that most of the RNAase is present in the free form, which is further supported by the absence of RNAase inhibitor in milk (see Table 5).

Alkaline RNAase inhibitor

When excess mammary alkaline RNAase inhibitor activity was measured

the highest levels were found in the lactating state (Table 5). Both the total amount of inhibitor per mammary gland and the specific activity was higher during lactation; the amount of inhibitor during early pregnancy was only 2-3% of that found during mid lactation. The inhibitor activity decreased upon ligation of the lactating gland. When the mammary gland homogenates were fractionated approximately 30% of the inhibitor activity was recovered in the cytosol (Table 5) and another 30% was found in the 15000xg pellet (not shown in the Table). Because of the lability of the inhibitor inactivation could not be avoided during the fractionation procedure. Insignificant amounts of the inhibitor sedimented with the microsomes (not shown). In liver tissue no differences were found regarding alkaline RNAase inhibitor activity between early pregnancy and mid-lactation. Three samples of milk were also investigated, but no inhibitor activity was detected.

DISCUSSION

In mammalian cells protein synthesis takes place on two morphologically and biochemically distinguishable structures namely on membrane-bound and free polysomes. It has been proposed that the membrane-bound polysomes are involved in the synthesis of proteins destined for export whereas free polysomes synthesize proteins for use in the cell (24,25). The proportion of free and membrane-bound polysomes differ widely depending on the type of cell studied. A typical example is mammary glands in which the two types of polysomes vary widely depending on the functional state of the gland. During lactation the gland is synthesizing large amounts of specific milk proteins on membrane-bound polysomes for nutrition of the young (3). Before this state is reached a phase of growth and development occurs. Then protein synthesis is mainly directed for production of proteins for internal use, and this synthesis takes place mostly on free polysomes. When litters are removed at weaning, emptying of the glands can not occur and involution together with a decrease in protein synthesis occurs. Events similar to those at weaning can be experimentally induced by ligation of the excretory ducts.

In a previous study a method for the extraction of free and membrane-bound polysomes was studied in rabbit liver tissue (4). The method was based on that described by Parish and Kirby (6). Contrary to their findings 28s RNA from membrane-bound polysomes could be extracted with PAS-TIPNS after prior extraction with NDS. When this sequential extraction procedure was used for the extraction of RNA from mammary glands a 28s RNA fraction could be extracted with PAS-TIPNS. The higher proportion of PAS-TIPNS extractable RNA in lactating glands compared to ligated glands or glands from pregnant rabbits also indi-

cates the higher proportion of membrane-bound polysomes in the lactating state.

The observed differences in the RNA content during pregnancy, lactation and ligation fit into a general pattern of RNA metabolism seen when RNAase and RNAase inhibitor studies were included from the same animals. Tissues with a low RNA content (i.e. pregnant or ligated mammary glands) showed high RNAase and low RNAase inhibitor activities whereas the reverse was found in mammary glands which have a high RNA content. These differences between a tissue of high RNA content (lactating glands) and one of a low RNA content (ligated glands) are illustrated in Fig. 3. The differences are also in agreement with the postulate by Kraft and Shortman (26) that there is a high inhibitor/RNAase ratio in tissues where an active protein synthesis takes place.

Concerning alkaline RNAase and its inhibitor our results are similar to those reported for rat mammary glands by Liu et al (13) who found the highest amount of alkaline RNAase inhibitor during lactation. They also observed a high RNAase activity at weaning, a functional state comparable to ligation. However, a higher amount of RNAase activity in rat mammary gland homogenates during lactation than during pregnancy has also been reported (27). In this case total RNAase activity was measured in the presence of Triton X-100 and PCMB was not included in the assay.

The changes of alkaline RNAase and its inhibitor observed during lactation compared to pregnancy might be due to the increase in protein content, especially milk proteins, in the glands during lactation. But since the specific alterations correspond to an increase in the inhibitor activity and a decrease in RNAase activity this would instead suggest a specificity in the observed changes. The milk contributed only with RNAase activity, but still, during lactation there

was an increase in alkaline RNAase inhibitor activity. This strengthens the specificity of the changes in RNAase and RNAase inhibitor system when lactating glands are compared to glands of pregnant rabbits. When liver tissue from pregnant and lactating rabbits were compared very small changes in specific activity of alkaline RNAase and RNAase inhibitor could be observed. These observations also support the idea of a specific change in the alkaline RNAase and RNAase inhibitor system in rabbit mammary glands during different physiological states. The present investigation extends the knowledge about the relationship between RNA, alkaline RNAase and its inhibitor in rabbit mammary glands during different experimental states. The findings indicate that an interplay by hormones and other physiological events influence mammary RNA metabolism.

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TABLE 1

Extraction of RNA from unfractionated mammary glands of two pregnant and two lactating rabbits. 1)

Functional state		RNA extracted (ODU ₂₆₀ per g tissue)	
		NDS	PAS-TIPNS
Pregnancy	1	35	1
	2	22	2
Lactation	1	107	19
	2	100	24

1)

Extraction was performed sequentially using NDS and phenol followed by PAS-TIPNS treatment of the insoluble interface.

TABLE 2

Recovery of RNA from microsomal fractions of rabbit mammary glands during lactation and ligation of the main excretory ducts on one side. Two animals were investigated and extracted sequentially with NDS and PAS-TIPNS.

Extraction agent	RNA extracted (ODU ₂₆₀ /g tissue)	
	Lactation	Ligation
<u>Rabbit 1</u>		
NDS	24.8	14.4
PAS -TIPNS	3.3	0.8
<u>Rabbit 2</u>		
NDS	60.0	26.0
PAS-TIPNS	10.0	3.0

TABLE 3

Distribution of RNA in microsomal subfractions from lactating and ligated mammary glands.

Lactating mammary glands:	RNA extracted ODU ₂₆₀ per g tissue
Membrane-bound polysomes	32.0
Free polysomes	3.6
Monosomes and subunits	6.7
Ligated lactating mammary glands:	
Membrane-bound polysomes	0.7
Free polysomes	8,7
Monosomes and subunits	13,4

The mammary ducts were ligated on one side on day 14 of lactation. Extraction of RNA with NDS and phenol-cresol followed by PAS-TIPNS was carried out 4 days later. The RNA extracted by the two procedures is given in the table.

Table 4

Amount and specific activity of alkaline RNAase in mammary glands and liver of pregnant rabbits and lactating rabbits with unilaterally ligated main excretory ducts. Meant SEM from four rabbits except the milk samples (three rabbits). Determinations were done in duplicate.

Mammary gland:	Homogenate		Cytosol			
	Total RNAase x 10 ³		Free RNAase x 10 ³		Total RNAase x 10 ³	
	Units per mammary gland	Spec.act.	Spec. act.	Spec. act.	Units per mammary gland	Spec.act.
Pregnancy	4 368 ± 67	6.6 ± 0.2	0.1		168 ± 18	8.8 ± 0.9
Lactation	2 1193 ± 550	1.9 ± 0.4	<0.1		920 ± 200	3.8 ± 0.7
Lactation/ Ligation	3460 ± 1200	3.4 ± 0.3	0.1		1585 ± 600	7.8 ± 1.2
Liver ³ :						0.45 ± 0.07
Pregnancy	-	2.1; 2.6	0:0		-	3.1; 3.7
Lactation	-	2.2; 2,6	0:0		-	3.4; 3.4
Milk	-	1.1 ± 0.1	0.9 ± 0.1		-	-

1) units/mg prot.

2) for details see Material and Methods

3) two livers from each group

4) eight or nine mammary glands from each rabbit

5) four or five mammary glands from each rabbit

Table 5

Amount and specific activity of excess alkaline RNAase inhibitor and inhibitor/alkaline RNAase ratio in mammary glands and liver of pregnant rabbits and lactating rabbits with unilaterally ligated main excretory ducts. The calculation of the ratios is based on the spec.act. given in table 1. Mean - SEM from four rabbits, except the milk samples (three rabbits). Determination were done in duplicate.

	Homogenate			Cytosol		
	Inhibitor x 10 ³			Inhibitor x 10 ³		
	Total units per mammary gland	Spec. act.	Ratio	Total units per mammary gland	Spec.act.	Ratio
Mammary glands:						
Pregnancy ⁴	135 ± 25	2.3 ± 0.3	0.35	46 ± 6	2.7 ± 0.4	0.31
Lactation ^{2,5}	5950 ± 54	4.1 ± 0.6	2.20	1820 ± 300	5.2 ± 0.5	1.40
Lactation/ Ligation	5100 ± 402	3.0 ± 0.9	0.89	1450 ± 303	3.0 ± 0.4	0.38
Liver ³ :						
Pregnancy	-	2.9; 3.7	1.38 ± 1.42	-	3.2; 3.5	1.03; 0.95
Lactation	-	3.3; 3.0	1.50; 1.15	-	3.1; 3.1	0.91; 0.78
Milk	0	0	-	-	-	-

1) units /mg prot.

2) for details see Material and Methods

3) two livers from each group

4) eight or nine mammary glands from each rabbit

5) four or five mammary glands from each rabbit

Whole tissue or subcellular fractions

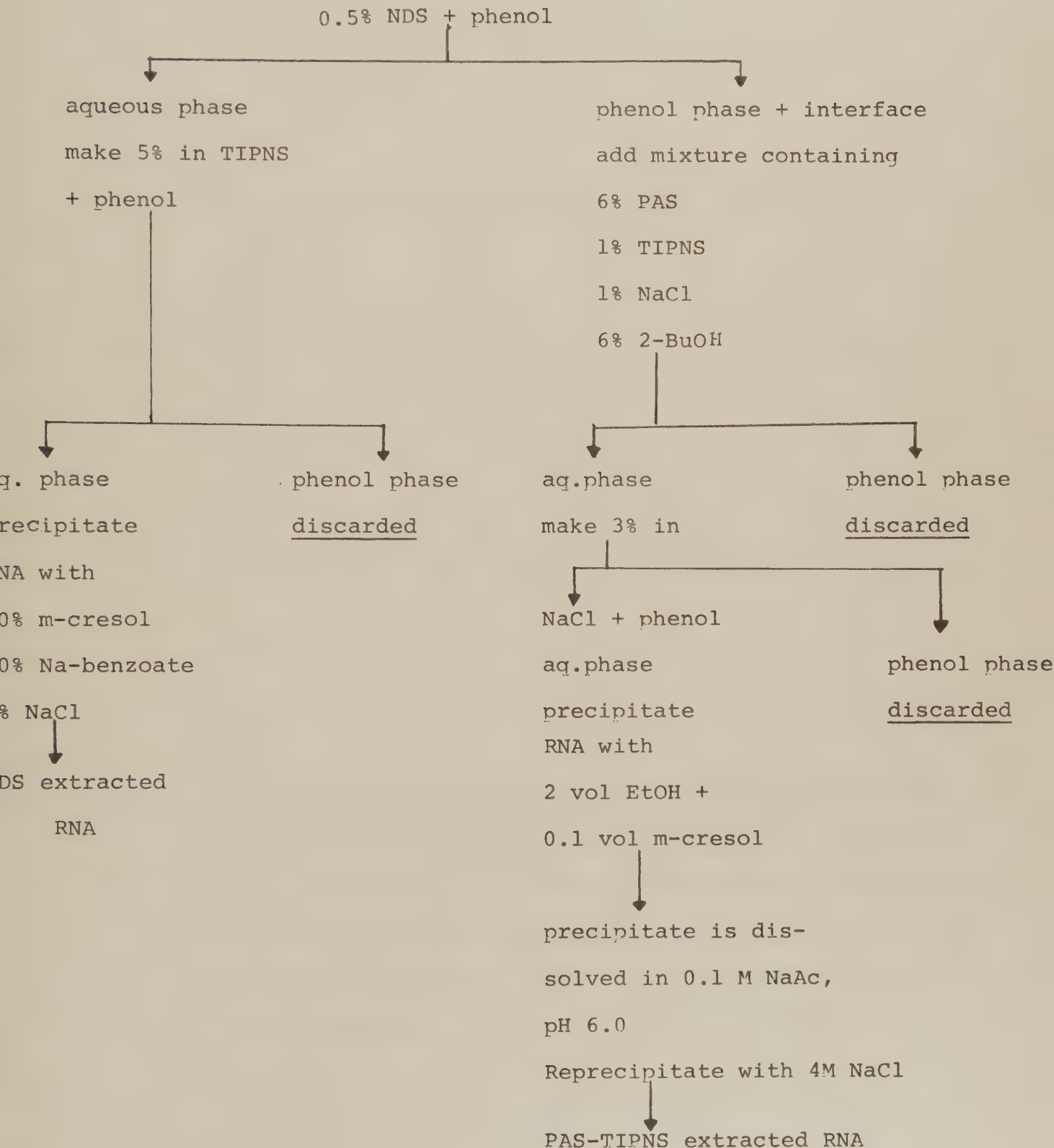


Fig 1. Sequential extraction procedure for RNA

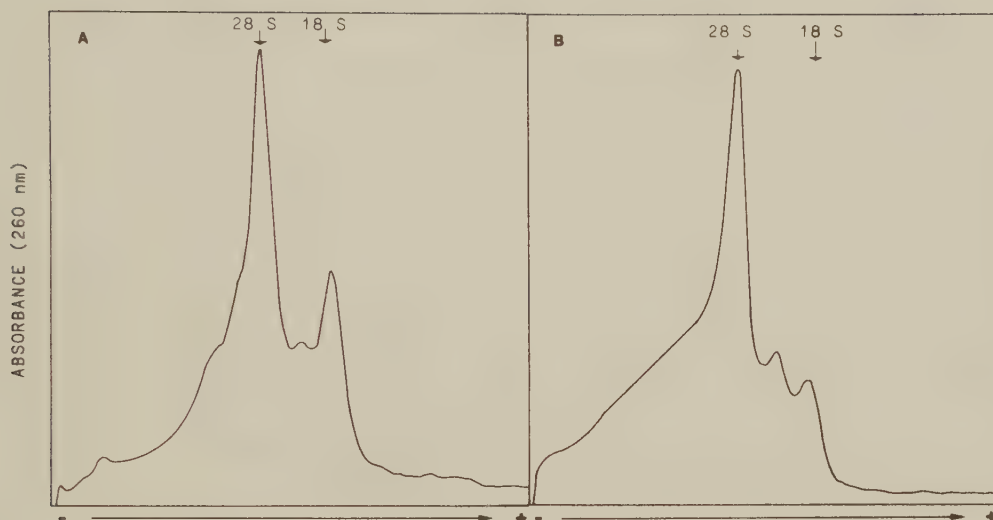


Fig. 2 . Polyacrylamide-agarose gel electrophoresis of microosomal RNA from lactating mammary glands sequentially extracted with A) naphthalene -1,5-disulphonate and B) 4-aminosalicylate plus triisopropyl naphthalene sulphonate. Electrophoresis was performed as described in Material and Methods. UV-absorbing material was monitored at 260 nm.

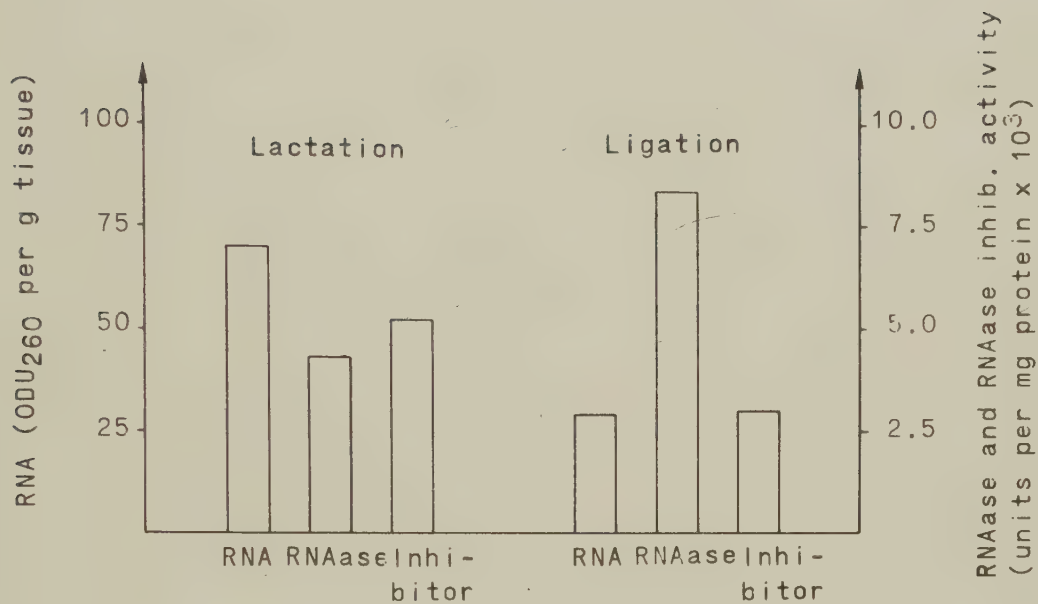


Fig. 3. Amount of RNA and activity of RNAase and RNAase inhibitor of mammary glands during lactation and after ligation of the main excretory ducts on one side. Ligation was performed between day 14 and 19 of lactation in local anesthesia.

TITLE: Separation of rRNA on Sepharose 2B at low ionic strength

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SUMMARY

A new method for the separation of 28s and 18s RNA has been examined. Rabbit liver rRNA was obtained by sequential extraction of the tissue with naphthalene -1,5-disulphonate (NDS) and phenol followed by 4-aminosalicylate and triisopropyl naphthalene sulphonate (PAS-TIPNS). The RNA was chromatographed on Sepharose 2B at low ionic strength with 1mM Mg^{2+} in the elution medium at 4°C. With both NDS- and PAS-TIPNS-extracted RNA a first included peak containing 18s RNA and a second well separated peak containing 28s RNA were obtained. By excluding Mg^{2+} from the medium or when the temperature was raised to 22°C in Mg^{2+} -containing medium an additional fraction of 28s RNA was eluted. PAS-TIPNS extracted RNA showed an additional chromatographic peak in the void volume containing 28s RNA which was due to the presence of salt in the extract. Thus, a separation of 28s and 18s RNA by chromatography on Sepharose 2B is best achieved by starting the elution at 1mM Mg^{2+} and 4°C to elute 18s RNA and in part 28s RNA and then remove Mg^{2+} from the medium to elute 28s RNA at the same temperature.

INTRODUCTION

Several methods have been used for the fractionation of nucleic acids, including gel filtration (1), gel electrophoresis (2,3), high speed centrifugation (4), selective salt precipitation (5) and affinity chromatography (6). Gel filtration of nucleic acids on agarose yields a good separation of DNA, rRNA and tRNA when the elution is carried out at a low ionic strength at +22°C (1). Although there is a great difference in molecular weight of 28s and 18s RNA these two RNA species can not be separated under these conditions. In order to separate 28s and 18s RNA on agarose other properties than molecular size must be considered. It has been demonstrated by other investigators (7-10) that a separation on agarose of these two RNA species is possible at high ionic strength (0.7 M NaCl). Thus, 28s RNA was retained on the agarose in an SDS-EDTA-Tris buffer in the presence of 0.7 M NaCl, while 18s RNA was eluted when the chromatography was carried out at 23-25°C. The retained 28s RNA could be eluted either by lowering the NaCl concentration to 0.1 M or by raising the temperature to 35°C.

We have previously shown (11) that it is possible to achieve a selective extraction of rRNA by the use of Kirby's extraction method (12). When naphthalene -1,5-disulphonate and phenol-cresol are used as extraction medium 18s RNA from free and membrane-bound polysomes and 28s RNA from free polysomes are extracted. A large part of the 28s RNA from membrane-bound polysomes is retained in the phenol phase by this treatment and is released into the aqueous phase when 4-amino-salicylate and triisopropyl naphthalene sulphonate are added.

We here describe the separation of 28s and 18s RNA on Sepharose 2B

at low ionic strength, starting the chromatography at $+4^{\circ}\text{C}$. Then 18s RNA is eluted plus one and sometimes two 28s RNA fractions well separated from the 18s RNA peak. By increasing the temperature to 22°C or omitting Mg^{2+} from the elution medium, an additional fraction containing 28s RNA is also obtained. The relationship between the three 28s RNA fractions will be discussed.

MATERIALS AND METHODS

Materials

Sepharose 2B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and 2% Gelarose from Litex Industries AS, Copenhagen, Denmark.

Methods

Fractionation of the tissue

Rabbits were killed by cervical dislocation and the livers were chilled immediately and rinsed in ice-cold buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$ and 0.25 M sucrose). The tissue was homogenized in a Potter-Elvehjem homogenizer and the homogenate was centrifuged at 10000xg for 10 min. The postmitochondrial supernatant was centrifuged at 105000xg for 3 h and the pellet (microsomes) was used for subsequent experiments.

Extraction of RNA

Extraction of RNA from microsomes was performed sequentially as described by Kirby (12) using naphthalene -1,5-disulphonate (NDS) and phenol-cresol in a first step followed by extraction of the insoluble interface with 4-aminosalicylate (PAS) and triisopropyl naphthalene sulphonate (TIPNS). An outline of the extraction is illustrated in Fig 1.

Gel chromatography

Sepharose 2B was packed in a Pharmacia column (1,5 x 65 cm). The agarose was allowed to sediment over night and was then washed with the elution buffer (2 mM sodium phosphate, pH 5.8, 1 mM $MgCl_2$ and 0.5% butanol) at a constant flow rate of 5 ml per h.

RNA samples (approximately 50 ODU₂₆₀ in 500 µl of buffer) were applied to the column at +4°C and fractions of approximately 4 ml were collected. The elution was then continued at 22°C. Absorbance of the effluent was recorded at 254 nm and the combined fractions from each RNA peak were precipitated overnight at -20°C with 0.1 vol of 17% sodium chloride and 2 vol of ethanol. The precipitate was collected by centrifugation at 10000xg for 5 min. The recovery of RNA was 80-90%.

Polyacrylamide gel electrophoresis

Mixed polyacrylamide-agarose gels were prepared according to Dingman and Peacock. (3). Polyacrylamide gels without agarose were prepared as follows. A separation gel with a total monomer concentration of 3% was used. The proportion of N,N'-methylenebisacrylamide was 14%. The total concentration of the acrylamide monomers of the spacer gel was 2,5% with a 15% proportion of N,N'-methylene-bisacrylamide. Gels of 7 cm length were prepared in glass tubes (inner diameter 0.5 cm) and polymerized as described by Loening (13). 0.5 ODU₂₆₀ of RNA was layered over the gels and the electrophoresis was for 75 min at 5 mA per tube at 20°C in 0.04 M Tris-HCl, pH 7.8, 0.02 M sodium acetate and 0.001 M EDTA. After the run the gels were placed in 1 M acetic acid for 15 min before staining for 1 h in 0.2% methylene blue dissolved in 0.2 M acetic acid and 0.2 M sodium acetate. Excess stain was removed by rinsing in several changes of water.

Zonal centrifugation

Linear 5-25% sucrose density gradients in 0.1 M sodium acetate, pH 5.0, were prepared in a Beckman Ti 14 rotor for preparative isolation of 28s and 18s RNA. The total gradient was composed of 135 ml overlay (0.1 M sodium acetate, pH 5.0), 20 ml of the RNA sample (400 ODU₂₆₀

in 0.1 M sodium acetate, pH 5.0, and 2.5% sucrose), 450 ml of a linear gradient (5-25% sucrose in 0.1 M sodium acetate, pH 5.0) and 155 ml displacement cushion (30% sucrose in 0.1 M sodium acetate, pH 5.0). The run was for 5 h at 45000 rpm at +10°C. After the run the gradient was unloaded and monitored at 254 nm through a Uvicord (LKB Beckman, Sweden). The 28s and 18s RNA fractions were collected and precipitated with 0.1 vol 20% sodium acetate and 2 vol ethanol.

RESULTS

The chromatographic behaviour on Sepharose 2B of 28s and 18s RNA separated by zonal centrifugation was first studied. For this purpose naphthalene -1,5-disulphonate extracted RNA from rabbit liver microsomes were run on a linear sucrose gradient (see Methods) and the isolated 28s and 18s RNA were analyzed by polyacrylamide gel electrophoresis to make sure that no degradation of the RNA occurred during the preparation. Fig. 2 (b and e) shows the purity of the two RNA fractions that were used for the chromatographic studies.

Approximately 50 ODU₂₆₀ of the isolated 28s RNA was applied to the Sepharose 2B column. From the elution profile (Fig. 3) is seen that small amounts of the RNA eluted at +4°C, while the retained RNA eluted when the temperature was raised to 22°C. When 18s RNA was chromatographed in the same manner, most of the applied material eluted at +4°C while the remaining RNA eluted when the temperature was raised to 22°C. The fractions from each peak were precipitated and analyzed by polyacrylamide gel electrophoresis. The two main peaks (i.e. 28s RNA eluted at 22°C and 18s RNA eluted at +4°C) showed the same electrophoretic mobility as the material applied to the chromatography column (Fig. 2 d and f). The two minor peaks did not show any distinct band on the gels. (Fig. 2c and g), indicating that some degraded products were obtained in these fractions. Evidently, when isolated 28s RNA and 18s RNA are chromatographed on Sepharose 2B, 18s RNA is eluted at +4°C and 28s RNA is retained on the column at that temperature but is eluted when the temperature is raised to 22°C. Thus these results show that it is possible to separate the two rRNA species when the chromatography is started at +4°C, whereas they are obtained together when the elution is started at 22°C (1).

The separation of RNA on Sepharose 2B was now extended to include total rRNA. The RNA was extracted from rabbit liver microsomes starting with naphthalene -1,5-disulphonate and phenol-cresol (NDS extracted RNA) followed by the addition of 4-aminosalicylate and triisopropyl naphthalene sulphonate to release additional RNA from the insoluble interface obtained in the first step (PAS-TIPNS extracted RNA). In a previous paper (11) we have shown that NDS extracted RNA contains 28s RNA, whereas the PAS-TIPNS extracted RNA mainly consists of 28s RNA. These two RNA extracts, differing in the 28s:18s RNA ratio, were chromatographically analyzed on Sepharose 2B in order to further characterize the two types of extracted RNA.

When NDS extracted RNA was chromatographed on Sepharose 2B, two included fractions (peaks 1 and 2) could be seen, when the elution was carried out at $+4^{\circ}\text{C}$ (Fig. 4). Peak 1 corresponds to the main fraction that was obtained when isolated 18s RNA was chromatographed alone. The second peak eluted at about one bed volume. After washing the column with an additional bed volume of elution buffer the temperature of the column was raised to 22°C . At this temperature the rest of the material eluted within the inclusion volume of the column (peak 3). The chromatographic pattern of the PAS-TIPNS extracted RNA (fig. 4) differed from that of the NDS extracted RNA. Peak 1 was less prominent in case, but the other two fractions found in the NDS extracted RNA were also found in PAS-TIPNS extracted material. In addition to these peaks, a new fraction appearing in the void volume emerged (fraction v_0).

The fractions corresponding to each peak were concentrated by ethanol precipitation and characterized by polyacrylamide gel electrophoresis. Densitometric tracings of the electrophoresis gels are shown in Fig. 5. The excluded material (v_0) which was only obtained from the PAS-TIPNS extracted RNA as well as peaks 2 and 3 from both extracts contain 28s RNA while peak 1 contains 18s RNA. Thus the elution of 18s RNA at $+4^{\circ}\text{C}$

and the retention of 28s RNA at this temperature, which could be shown when they were chromatographed separately, also appeared when unfractionated rRNA was studied. But in addition to these two fractions, two new peaks were found.

The appearance of fraction v_0 is probably a consequence of the presence of salt in PAS-TIPNS extracted rRNA due to the extraction procedure. The last step of the isolation procedure involved a wash of the RNA with 4 M NaCl in 0.1 M NaAc, pH 6.0, after which the precipitate was dissolved in water. When 50 ODU₂₆₀ RNA extracted with PAS-TIPNS were dialysed against 2mM sodium phosphate, pH 5.8 prior to chromatography, the excluded material decreased from 7,6 ODU₂₆₀ to 1,3 ODU₂₆₀ compared to the undialysed material and this RNA was retained on the column. In an additional experiment salt was added to the dialysed material (0.2 M NaCl and 0.001 M MgCl₂) and the excluded peak reappeared. It is evident from these findings that it is important to keep the ionic strength low when the chromatography is carried out at +4°C in the presence of Mg²⁺, in order to avoid the fraction which can appear in the void volume.

The presence of Mg²⁺ is an absolute requirement for obtaining separation of 28s and 18s RNA at low ionic strength. When Mg²⁺ is omitted, no material is retained on the column. In the absence of Mg²⁺, 95% of the recovered RNA was obtained as a peak (corresponding to peak 1) at +4°C and only 5% was retained on the column. In the presence of Mg²⁺ the normal chromatographic pattern was obtained, which means that about 30% of the applied material was recovered in peak 1. Therefore, in order to investigate the possibility to elute the retained material by omitting Mg²⁺ instead of raising the temperature to 22°C the following experiment was performed. The same amount of RNA (50 ODU₂₆₀) was applied to two Sepharose 2B columns and after elution at +4°C in the presence of Mg²⁺ the elution of retained material was continued in two ways. In one case

the temperature was raised to 22°C, while in the other case Mg^{2+} was omitted from and 2mM EDTA was included in the elution buffer (Fig.6). In both these cases the retained RNA was eluted, but with EDTA in the buffer a very sharp peak appeared after one bed volume of effluent compared to the very broad peak seen when an increase in temperature was used for the elution of retained RNA.

Discussion

Sephadex is known to separate molecules according to size. This is known for DNA, 4s and 5s RNA, mRNA and viral RNA (1,14). Separation of 28s and 18s RNA at low ionic strength in the presence of Mg^{2+} at $22^{\circ}C$ yields the two RNA species as one peak, although they have a great difference in molecular weight (1). The present results show that it is possible to obtain a separation of 28s and 18s RNA if the chromatography instead is started at $4^{\circ}C$ when 18s RNA is eluted as a single peak. The 28s RNA behaves differently. While a part of this RNA usually is eluted as a peak well separated from the 18s RNA at $4^{\circ}C$, a sizeable part is retained on the column under these conditions. The retained RNA is released from the column either at $4^{\circ}C$ if Mg^{2+} is removed from the elution buffer or by raising the temperature to $22^{\circ}C$. Our finding that 28s RNA is retained on Sephadex 2B under certain conditions is comparable to other observations (7-10) where the same effect was shown in the absence of Mg^{2+} using high monovalent cation concentrations and at a temperature of $22-25^{\circ}C$. It is evident that the elution behaviour of 18s RNA is less dependent on ionic and temperature conditions than is that of 28s RNA.

The fact that 18s RNA elutes in front of the part of 28s RNA which is not retained on the Sephadex column (and not the reverse which is expected from their respective sizes) together with the retention of a large fraction of 28s RNA suggests that there are other and stronger interactions between 28s RNA and Sephadex than between 18s RNA and Sephadex. The elution of 28s RNA as a sharp peak when Mg^{2+} is removed from the medium may indicate that ionic pairs are formed between negatively charged groups on Sephadex and RNA mediated via

Mg^{2+} . Several batches of Sepharose 2B have been examined and the retention capacity differs from one batch to another. If this is low (i.e. small amount of RNA in peak 3) the partially retained fraction (peak 2) increases and vice versa. Gelarose (2%) was also studied and the same results as described for Sepharose were found.

It is of importance that the RNA that is applied to Sepharose 2B is free of monovalent cations to avoid the appearance of an additional fraction in the void volume. No difference between this fraction and the other two fractions containing 28s RNA could be detected.

The relative amounts of 28s and 18s RNA from NDS and PAS-TIPNS extracted RNA, respectively, prepared in the sequential manner were compared using the Sepharose 2B separation technique. The NDS extracted RNA which has been shown to have a 28s:18s RNA ratio of around 2.5:1 on polyacrylamide gel electrophoresis (11) gave a chromatography profile in accordance to this with about 30% of the recovered RNA being 18s RNA (fig 1. peak 1). This peak was very small in PAS-TIPNS extracted RNA, which has been shown earlier to consist mainly of 28s RNA (11).

The present investigation has shown that it is possible to separate 28s and 18s RNA on Sepharose 2B at low ionic strength at 4°C. The possibility to perform the chromatography at low temperature makes the technique advantageous, since degradation of RNA molecules might occur when the separation is done at higher temperatures.

Acknowledgments

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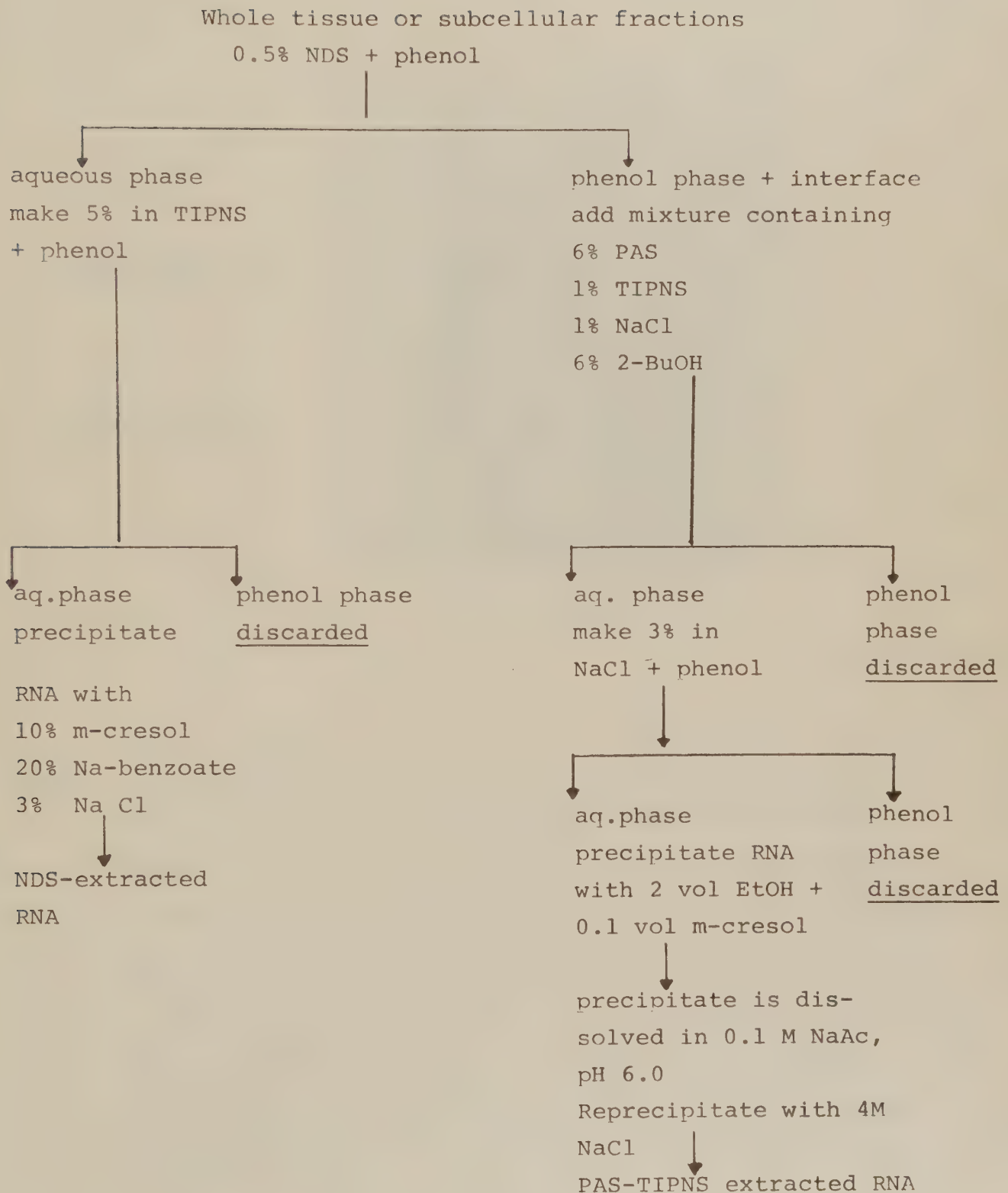


Fig. 1 Sequential extraction procedure for RNA.

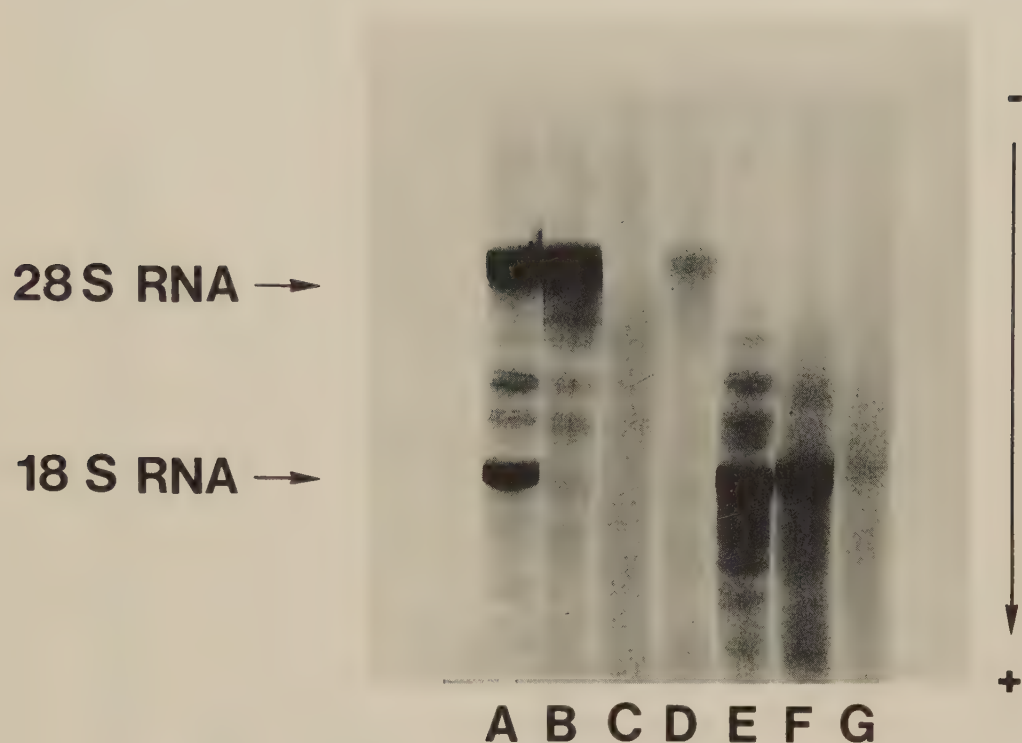


Fig 2. Polyacrylamide gel electrophoresis of isolated 28s and 18s RNA chromatographed on Sepharose 2B after prior separation of the RNA species by zonal centrifugation. Electrophoresis was performed as described in Material and Methods. A; unfractionated RNA, B; 28s RNA isolated by zonal centrifugation, C: 28s RNA eluted at 4°C, D; 28s RNA eluted at 22°C, E: 18s RNA isolated by zonal centrifugation, F; 18s RNA eluted at 4°C, F: 18s RNA eluted at 22°C.

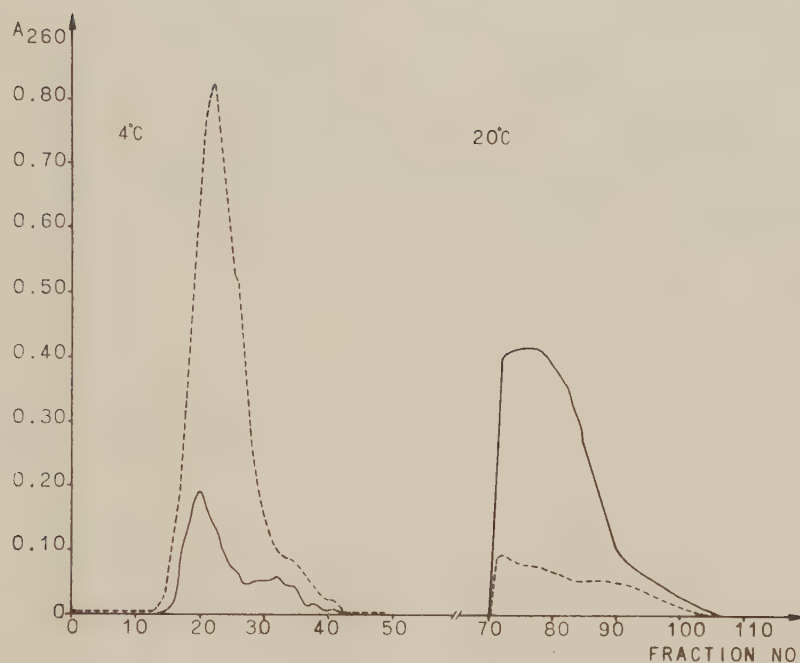


Fig 3 Chromatography of 28s and 18s RNA on Sepharose 2B column (1.5x 60 cm). The flow rate was 5 ml per hour and fractions of 3.8 ml were collected. The column was developed with 2mM sodium phosphate, pH 6.0, 1mM magnesium chloride and 0.5% butanol. The separation was first performed at 4°C and after elution with 2 bed volumes the temperature was raised to 22°C. ----- 18s RNA; _____ 28s RNA.

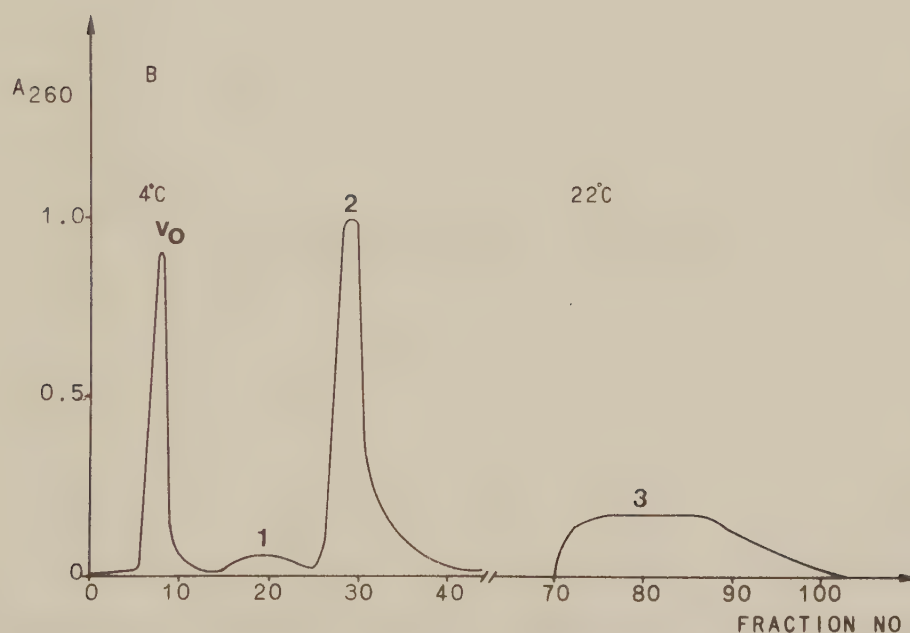
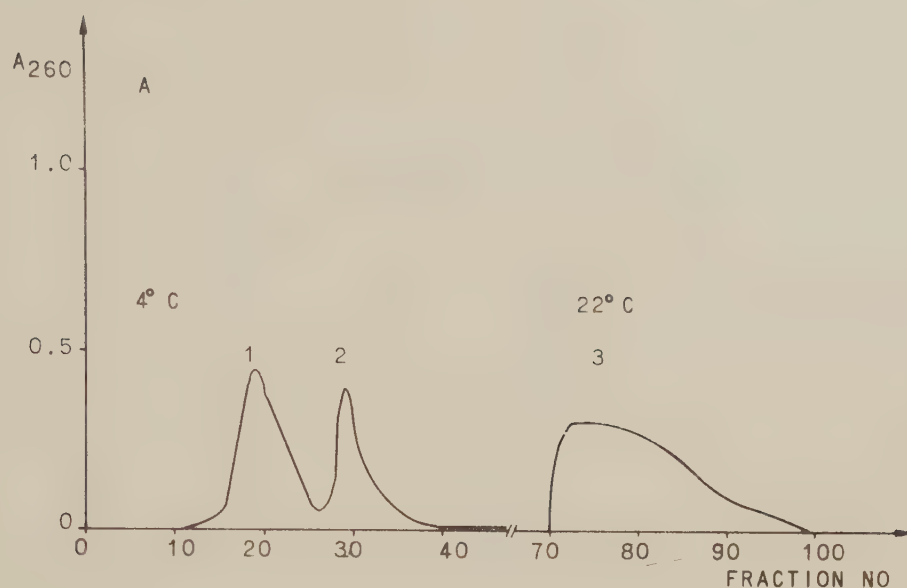


Fig 4. Chromatography of NDS and PAS-TIPNS extracted RNA on Sepharose 2B column (1.5 x 60 cm). The separation was first performed at 4°C and after elution with 2 bed volumes the temperature was raised to 22°C. The flow rate was 5 ml per hour and fractions of 4.3 ml were collected. The column was developed with 2 mM sodium phosphate, 1 mM magnesium chloride and 0.5% butanol. - A: -NDS extracted RNA; B: PAS-TIPNS extracted RNA.

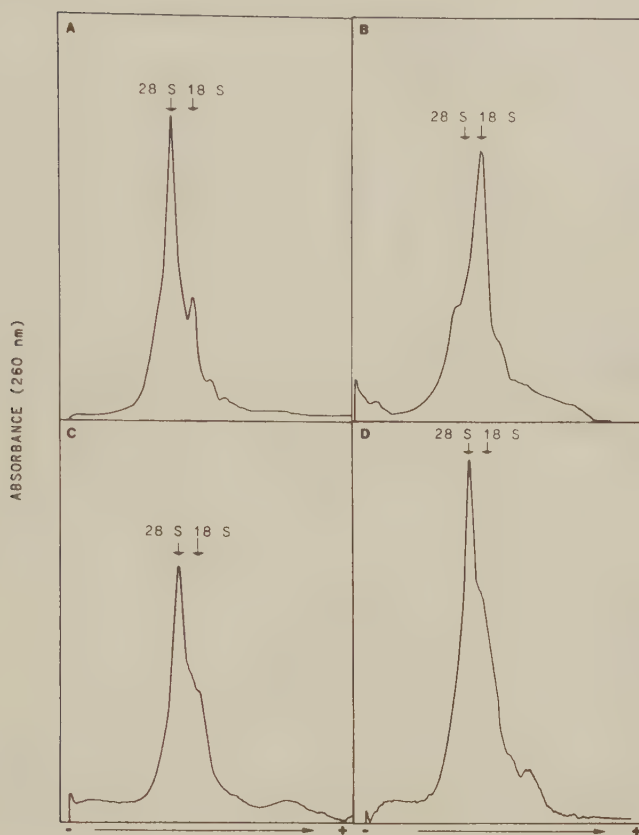


Fig 5 Polyacrylamide-agarose gel electrophoresis of RNA fractions obtained from Sepharose 2B chromatography.

Electrophoresis was performed as described in Materials and Methods. After the run UV-absorbing material of the gels were monitored in an Acta III Beckman Spectrophotometer at 260 nm. A: v_0 (void fraction), B: peak 1, C: peak 2, D: peak 3 (see fig 4).

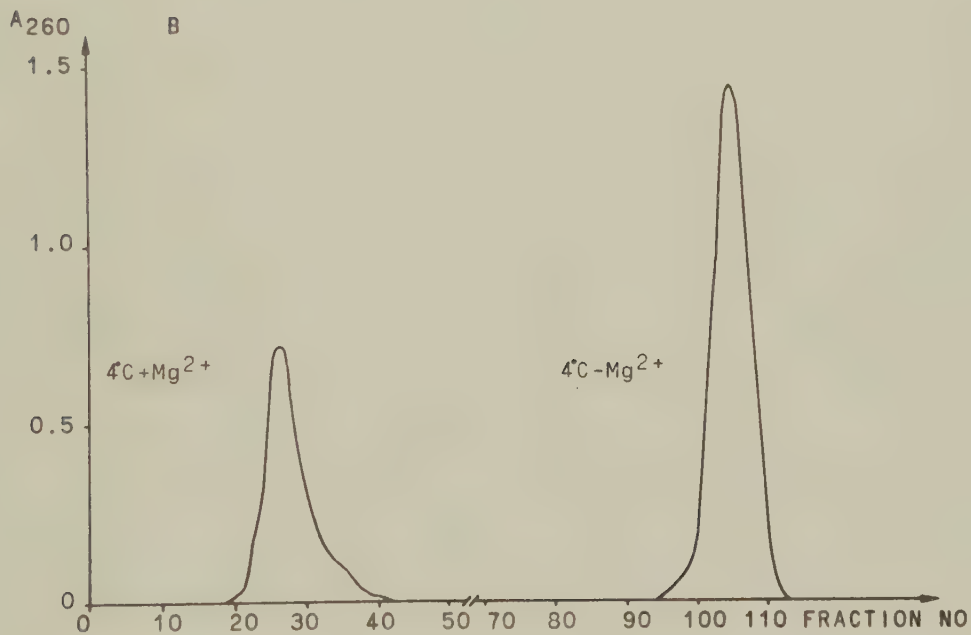
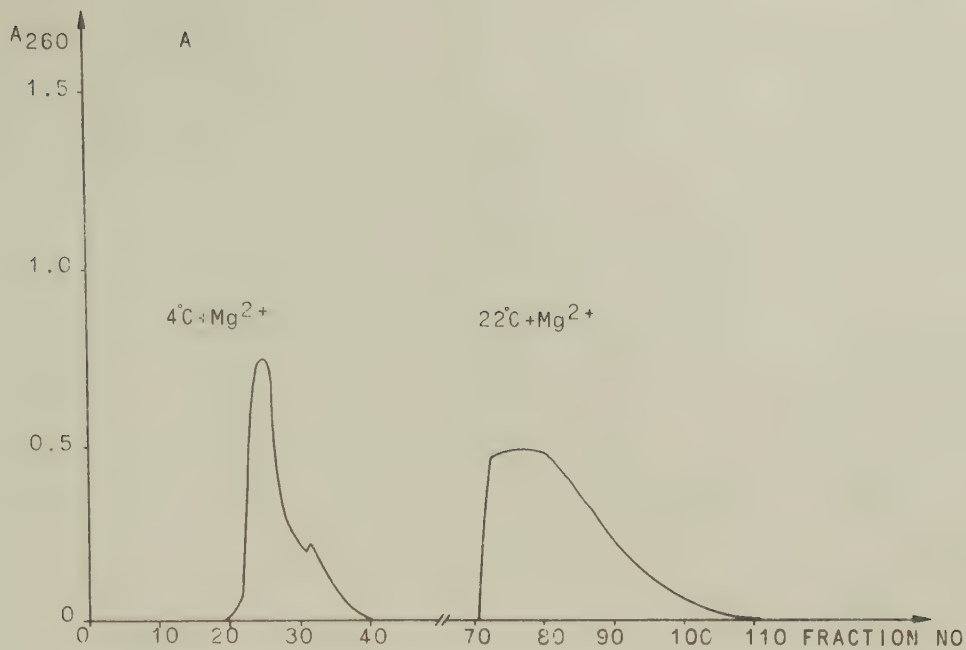


Fig.6. Chromatography of NDS extracted RNA on Sepharose 2B column (1.5 x 60 cm). The separation was first performed at 4°C in 2mM sodium phosphate, 1mM magnesium chloride and 0.5% butanol. After the elution had proceeded to 2 bed volumes either the temperature was raised to 22°C (A) or magnesium was excluded (B). The flow rate was 5 ml per hour and fractions containing 5 ml were collected. See text for further details.

PREPARATION OF POLY (A)-BINDING PROTEINS FROM ENDOPLASMIC RETICULUM-CONTAINING SUBFRACTIONS OF RAT LIVER CELLS AND THEIR USE IN mRNA PURIFICATION

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Summary

Approximately 2% of the proteins solubilised from rat liver microsomes or rapidly sedimenting endoplasmic reticulum (RS-ER) adsorbed to poly(A)-Sephadex. The adsorption appeared to be selective for a few proteins, and proteins of different apparent molecular weights adsorbed from RS-ER and the microsomes. The proteins from RS-ER with affinity for poly(A) were coupled to Sephadex and used for the purification of mRNA from rabbit mammary glands. A portion of the RNA which did not adsorb to poly(U)-Sephadex adsorbed to protein-Sephadex and was active in a cell-free protein synthesis system.

Introduction

A large proportion of mRNA molecules in eucaryotic cells possess a 3'-terminal poly(A) segment of 150–200 nucleotides, which appears to be coupled to the mRNA before it is transported from the nucleus into the cytosol¹. The poly(A) segment of this mRNA may participate in the formation of mRNP (ribonucleoprotein) particles together with specific soluble proteins². It may also be involved in the attachment of mRNA to intracellular membranes^{3,4}.

By using poly(A)-Sephadex as an adsorbent, proteins have been prepared from the nucleosol

and cytosol of rat liver cells and from the cytosol of HeLa cells^{5,6}. It is possible that proteins which will bind to poly(A)-Sephadex will also bind to poly(A) segments of mRNA (or HnRNA) in the cell.

We wish to report on the selective adsorption of proteins solubilised from two different fractions of rat liver endoplasmic reticulum (ER), rapidly sedimenting ER (RS-ER) and the microsomes to poly(A)-Sephadex. These proteins have been immobilised on Sephadex and used for the purification of mRNA from lactating rabbit mammary glands.

Materials and Methods

RS-ER and microsomes were prepared as described earlier⁷ from livers of Sprague-Dawley rats. All preparations were performed at 4 °C. The cytosol fraction was obtained as the post-microsomal supernatant. RS-ER and microsomes were washed with buffer A (50 mM Tris-HCl, pH7, 6; 8 mM MgCl₂; 0.225 M KCl; 0.35 M sucrose) before solubilisation in 1% Triton X-100 (the Triton X-100: protein ratio was 2:1 by weight) in the buffer. After centrifugation at 90,000 g for 2.5 h, ammonium sulfate was added to the supernatant to a saturation of 60%. After centrifugation, the floating membrane proteins were collected and dialysed overnight against buffer B containing 10 mM Tris-HCl, pH7.4; 10 mM NaCl; 1.5 mM MgCl₂; 5% (w/v) glycerol; 1 mM 2-mercaptoethanol; 0.05% (w/v) sodium

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deoxycholate. Dialysed samples were clarified by centrifugation and kept at -90°C until use.

Poly(A)-Sephacrose was prepared essentially as described by WAGNER⁸. The BrCN-activated Sephacrose was stirred gently with 2 mg per ml gel of potassium poly(A) (Sigma Chemical Co.) at 4°C for 15 h either in a 10 mM potassium phosphate buffer, pH7.5, or in a 0.2 M morpholinoethane sulfonate (MES) buffer, pH6.0. Control Sephacrose gels were prepared by using the same procedure as above but omitting the poly(A). When coupling was performed in the phosphate buffer, a much higher degree of poly(A) substitution was obtained than in the MES buffer (0.58 mg poly(A) coupled per ml gel compared to 0.34 mg, corresponding to coupling yields of 27% and 16%, respectively). Gels prepared in phosphate buffer were used for the experiments except when otherwise stated, since such gels consistently adsorbed more proteins than did gels prepared in MES buffer (cf. Table 1).

Protein-Sephacrose was prepared by using protein samples from RS-ER. Solubilised proteins which did not adsorb on control gels were applied on poly(A)-Sephacrose. The protein fraction which eluted with 0.1 M KCl was concentrated by ultra-filtration and chromatographed on a Sephadex G-50 (Pharmacia) column to remove possible contaminating ribonuclease activity. The fractions of the void volume were concentrated and dialysed overnight against 0.1 M sodium bicarbonate, pH8.5. The proteins were coupled to BrCN-activated Sephacrose (50 mg BrCN/ml gel) at 4°C for 15 h⁹. The protein-Sephacrose gels were thoroughly washed with 0.1 M sodium bicarbonate, 1 M KCl in buffer B, and buffer B before use.

SDS-polyacrylamide gel electrophoresis was performed in slab gels in a Pharmacia GR 4 apparatus¹⁰. Gels were stained with 0.1% Coomassie Brilliant Blue and destained in 25% methanol- 7% acetic acid. Gels with radioactive samples were dried under vacuum at 100°C and autoradiographed using Agfa T 4 films; the exposure was for 1-3 days.

RNA was prepared from rat livers and lactating rabbit mammary glands¹¹. Poly(A)-containing mRNA was purified on poly(U)-Sephacrose as described by LINDBERG and PERS-SON¹². *In vitro* cell-free protein synthesis was performed using wheat germ extracts¹³. Protein

concentrations were determined according to LOWRY *et al.*¹⁴

Experimental and Discussion

Poly(A)-Sephacrose chromatography

When proteins from rat liver cytosol, or proteins solubilised from RS-ER or microsomes, were applied to poly(A)-Sephacrose columns, a significant proportion of the proteins were adsorbed. These proteins could be desorbed with 0.1 M KCl, and constituted 2-13% of the applied sample (the amount depended on the origin of the sample and the buffer used during the substitution of the gels—cf. Table 1). To test whether proteins bound nonspecifically to Sephacrose due to changes in the gel caused by the activation procedure, samples from the three rat liver fractions were applied to control Sephacrose. A significant proportion (2-8%, depending on the fraction applied) of the proteins were adsorbed, and could be eluted with 0.1 M KCl (Table 1). Nonadsorbed material was in turn applied to poly(A)-Sephacrose, which adsorbed another fraction of the proteins. These proteins could be eluted with 0.1 M KCl; only trace amounts of proteins were released when KCl concentrations were raised to 1 M. The proportion of proteins with affinity for poly(A)-Sephacrose was slightly higher in RS-ER than in microsomes while the control Sephacrose adsorbed more microsomal proteins. The proportion of proteins adsorbed from the cytosol fraction was much higher than from the membrane fractions, both on control gels, and on poly(A)-Sephacrose.

Table 1. Adsorption of proteins to poly(A)-Sephacrose and control Sephacrose.

Sample	Phosphate buffer		MES buffer	
	Control Sephacrose	Poly(A)-Sephacrose	Control Sephacrose	Poly(A)-Sephacrose
RS-ER	2.1	2.2	2.6	1.3
Microsomes	3.1	1.7	1.4	0.8
Cytosol	7.9	5.5	3.6	2.5

Between 7 and 10 mg protein of each sample was chromatographed on control Sephacrose and the nonadsorbed peaks were rechromatographed on poly(A)-Sephacrose. The figures represent the amount (in %) of applied protein adsorbed on gels prepared in phosphate or MES buffer subsequently eluted with 0.1 M KCl.

Table 1 also shows data on the affinity of proteins for gels prepared in MES buffer which is often used^{5,6} during the coupling of poly(U) or poly(A) to Sepharose. The yields of adsorbed proteins were much lower using such gels.

SDS-polyacrylamide gel electrophoresis

The protein peaks obtained with 0.1 M KCl from poly(A)-Sepharose and control gels were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). The material applied to the control gels was also electrophoresed as reference. Material from all the three liver fractions eluted with 0.1 M KCl contained several proteins, a few of which were particularly enriched in the eluates from poly(A) gels.

The most prominent proteins in the eluate of the RS-ER extract from poly(A)-Sepharose had apparent molecular weights of 54,000, 56,000, 79,000 and 130,000 d (as indicated in the figure). The 56,000 and 130,000 d proteins, however, were also present in the eluate from

the control gel and their adsorption might not be specific for the poly(A) segment. In the eluate of the microsomal extract several proteins were seen which also adsorbed to the control gel. The proteins of apparent molecular weights 57,500, 60,000, 63,000 and 79,000 d, however, seemed to be adsorbed preferentially to the poly(A)-containing gel. The cytosol eluate showed one major protein (apparent molecular weight 43,000 d) with higher affinity for the poly(A) gel than for the control gel. The 79,000 d protein in the membrane fractions might be identical to the protein of similar molecular weight isolated by BLOBEL¹⁵ from rat hepatocyte mRNA particles which was suggested to bind specifically to the poly(A) region of mRNA. SCHWEIGER and MAZUR⁶ have among other proteins also identified a 43,000 d protein with affinity for poly(A)-Sepharose.

From these experiments two conclusions can be drawn. One is that there are membrane proteins in RS-ER and microsomes which seem

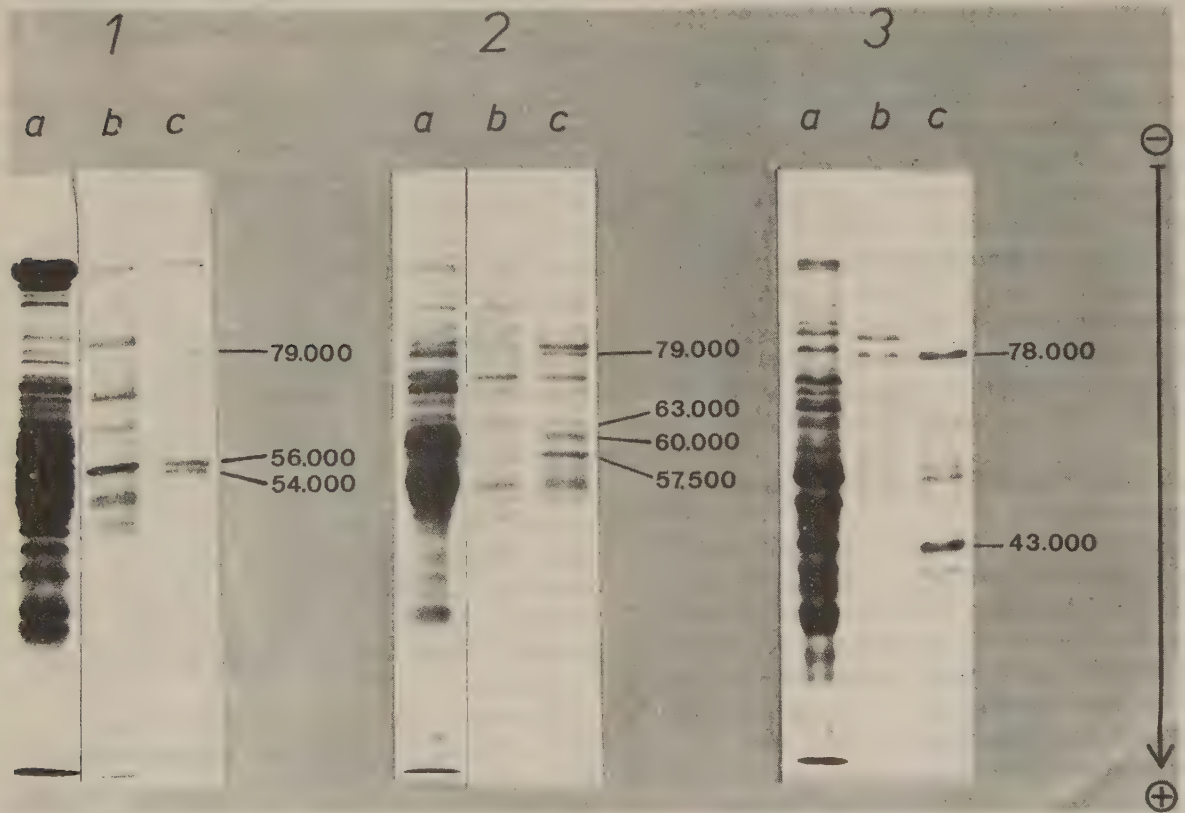


Fig. 1. SDS-gel electrophoresis of proteins from RS-ER (position 1), microsomes (position 2) and cytosol (position 3). a, crude extracts; b, protein adsorbed on control Sepharose; c, proteins adsorbed on poly(A)-Sepharose. The following molecular weight standards were used: bovine serum albumin, ovalbumin, soybean trypsin inhibitor and cytochrome c. The composition of the gel was 11.1 × 0.9¹⁷.

to have affinity for poly(A) sequences and therefore can be enriched by chromatography on poly(A)-Sepharose. A comparison between the electrophoretic patterns of such proteins from RS-ER and microsomes shows that the proteins are specific for each membrane fraction and therefore might reflect differences both in function and protein composition between these two fractions. Furthermore, the proteins differ from the cytosol proteins that have affinity for poly(A)-Sepharose.

Another conclusion to be drawn is that some proteins have an affinity for the control Sepharose. It is therefore important to remove such proteins before application of protein samples to the derivatised Sepharose to avoid nonspecific adsorption due to changes in the gel introduced by the activation procedure. Accordingly, when samples were applied directly to poly(A)-Sepharose and eluted with KCl the composite protein pattern of eluates from poly(A) gels and control gels was obtained.

A possible function of membrane proteins with affinity for poly(A) is in the binding of mRNA to the ER. This should be in line with the model where mRNA participates in the ribosome-membrane interaction as has been suggested for rat liver³. The observation that different proteins from RS-ER and microsomes adsorb to poly(A)-Sepharose might indicate that different ribosome-membrane binding mechanisms operate in these two fractions. A further indication for different binding mechanisms in these fractions is the isolation of cytochrome P-450-ribosome complexes upon detergent treatment of RS-ER but not of microsomes¹⁶.

Preparation of mRNA by protein-Sepharose chromatography

To examine whether proteins with affinity for poly(A)-Sepharose can be used as adsorbents for RNA (in particular mRNA) as an alternative to poly(U)-Sepharose, such proteins prepared from RS-ER were coupled to Sepharose by the BrCN method. Samples of RNA obtained from rabbit mammary glands and rat liver were chromatographed on protein-Sepharose and, for comparison, on poly(U)-Sepharose. The RNA from mammary glands that did not adsorb on the poly(U) gels was collected and rerun on protein-Sepharose to test whether a specific fraction of the RNA could then be obtained.

Table 2. Adsorption of rabbit mammary gland RNA to poly-(U)-Sepharose and protein-Sepharose, and its mRNA activity.

Preparation method	Amount of RNA adsorbed (μg)	(%)	Specific protein synthesis (CPM/μg RNA)	Total protein synthesis (CPM)
Poly(U)-Sepharose	400	4.6	1.29 x 10 ⁵	5.2 x 10 ⁷
Protein-Sepharose	650	7.4	0.79 x 10 ⁵	5.1 x 10 ⁷
Protein-Sepharose (nonadsorbed RNA from poly(U)-Sepharose applied)	500	5.9	0.33 x 10 ⁵	1.7 x 10 ⁷

Postmitochondrial RNA from lactating mammary glands (8.75 mg) was applied to poly(U)-Sepharose or protein Sepharose columns prepared as described in Materials and Methods. Adsorbed RNA was eluted with 0.1 M KCl. Nonadsorbed RNA was rerun on protein-Sepharose. The figures given are in % of the originally applied sample. The values for the amount of RNA adsorbed might include some ribosomal RNA which is not removed by a single passage through the columns. Since there might be trace amounts of ribonuclease in the protein sample coupled to Sepharose a second passage through the column was omitted. Protein synthesis was performed with 5 μg of mRNA using ³⁵S-methionine as the labelled aminoacid.

The data is collected in Table 2. Of the applied RNA from mammary glands, 7.4% adsorbed on protein-Sepharose and was eluted with 0.1 M KCl, compared to the 4.6% adsorbed on poly(U)-Sepharose. When this RNA was tested in a cell-free protein synthesising system, it showed a high specific messenger activity, and the total activity of the RNA eluted from the protein-Sepharose was comparable to that eluted from poly(U)-Sepharose. The non-adsorbed peak from the poly(U) gel contained RNA which also showed messenger activity with affinity for protein-Sepharose (5.9% of the original sample). Preliminary experiments suggest that a fraction of rat liver RNA with messenger activity will adsorb on the protein-Sepharose in a similar way, and that this fraction is larger than that adsorbed on poly(U)-Sepharose.

Thus our results show that proteins with affinity for poly(A)-Sepharose can be used for the purification of mRNA and, furthermore, that a fraction of mRNA which is not adsorbed on poly(U)-Sepharose can be purified on the protein-Sepharose gel. The basis for this discrimination between mRNA populations is not known, but we are examining whether, for



Fig. 2. Autoradiographs of ^{35}S -methionine-labelled proteins separated by SDS-gel electrophoresis. Samples were obtained by *in vitro* protein synthesis using mRNA prepared from lactating rabbit mammary glands. a, RNA adsorbed on poly(U)-Sephadex; b, RNA adsorbed on protein-Sephadex; c, control without added RNA. The composition of the gel was 13.1×1.1^{17} .

instance, the size of the 3'-terminal poly(A) segment might determine to which adsorbent the mRNA will bind.

The products of the cell-free protein synthesis using mammary gland RNA adsorbed on poly(U)-Sephadex and protein-Sephadex were examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 2). The most prominent distinct products show the same molecular weight distribution in the two cases. The molecular weights were in the range of 15,000–35,000 d corresponding to the range of the principal milk proteins. Presently we are investigating whether different populations of mRNA from a less specialised tissue than mammary glands will bind to poly(U)-Sephadex than to protein-Sephadex, and whether the protein-Sephadex can be used for the purification of specific mRNA species.

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